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have invented new and useful improvements in:

Isolation of Nucleic Acids on Surfaces

of which the following is a specification

Isolation of Nucleic Acids on Surfaces

Field of the Invention

This invention concerns new processes for the isolation and purification of nucleic acids on surfaces.

Cross-reference to Related Applications

This application is a continuation-in-part of pending International application no. PCT/EP99/02664, filed April 20, 1999 and designating the United States, and of pending International application no. PCT/EP98/06756, filed October 23, 1998 and designating the United States and claiming priority to German application DE 19746874.8, filed October 23, 1997.

Background of the Invention

It has been known for a long time that the genetic origin and functional activity of a cell can be determined and studied by examination of its nucleic acids. Methods of analyzing nucleic acids permit direct access to the cause of cell activity. Such methods are therefore potentially superior to indirect conventional methods such as detecting metabolic products. For that reason a large expansion in the number of nucleic acid analyses can be expected in the future. For instance, molecular biological analyses are already used in many areas, for example, in medical and clinical diagnostics, in pharmacology for the development and evaluation of medications, in analysis of foodstuffs as well as monitoring food manufacturing and food inspection, in the agricultural business for breeding useful plants and animals, in environmental analysis, and in

many research areas, including, for example, paternity analyses, tissue typing, identification of genetic diseases, genome analyses, molecular diagnostics, such as the identification of infectious diseases, transgenic research, basic research in the area of biology and medicine, as well as in numerous related areas.

Through RNA analysis, especially mRNA in cells, gene activity can be determined directly. The quantitative analysis of transcript patterns (mRNA patterns) in cells, by way of modern molecular biology methods, such as, e.g., real-time reverse transcriptase PCR ("Real-Time RT-PCR") or gene expression chip analyses, permit for example the recognition of defectively expressed genes, through which many types of disorders, e.g., metabolic diseases, infections or the generation of cancer, may be recognized. Analysis of DNA from cells by way of molecular biological methods, such as, e.g., PCR, RFLP, AFLP or sequencing, permits for example the assessment of genetic defects in or the determination of the HLA type as well as of other genetic markers.

The analysis of genomic DNA and RNA is also utilized to directly prove the existence of infectious stimuli, such as viruses, bacteria, etc.

In this connection, a general difficulty exists in the fact that biological and/or clinical samples must be prepared in such a way that the nucleic acids contained therein can be utilized directly in the analytical method in question. It is especially important that the nucleic acids be provided in good yield, that the recovered nucleic acids be of high quality, and that there be high reproducibility, in particular where there are a greater number of samples, in which case the analysis should be capable of being conducted automatically.

The state of the art already includes many processes for the purification of DNA. For example, it is known how to purify plasmid DNA for the purpose of cloning and other experimental processes. See, e.g., the method of Birnboim, *Methods in Enzymology*, 100:243 (1983). In this process, a cleared lysate of bacterial origin is exposed to a cesium chloride gradient and centrifuged for a period of 4 to 24 hours. This step is usually followed by the extraction and precipitation of the DNA. This process is associated with the disadvantages that it is very apparatus-intensive, and it takes a great deal of time, is expensive to run and cannot be automated.

Other methods in which cleared lysates are used to isolate DNA are based on ion-exchange chromatography (e.g., Colpan et al., *J. Chromatog.*, 296:339 (1984)) and gel filtration

(e.g., Moreau et al., *Analyt. Biochem.*, 166:188 (1987)). These processes are primarily alternatives to the cesium chloride gradients; however they require an extensive solvent supply system, and a precipitation of the DNA fractions is necessary, since these usually contain salts in high concentrations and are extremely diluted solutions.

Marko et al., *Analyt. Biochem.*, 121:382 (1982), and Vogelstein et al., *Proc. Nat. Acad. Sci.*, 76:615 (1979), have found that if the DNA from extracts containing nucleic acids is exposed to high concentrations of sodium iodide or sodium perchlorate, only DNA will adhere to glass scintillation tubes, fiberglass membranes or fiberglass sheets that have been finely ground by mechanical means, while RNA and proteins will not. The DNA that has been bound in this manner can be eluted, for example, with water.

For example, in international publication WO 87/06621, the immobilization of nucleic acids on a PVDF membrane is described. However, the nucleic acids bound to the PVDF membrane are not eluted in the next step; instead the membrane, together with all the bound nucleic acids is introduced directly into a PCR reaction. Finally, in this international patent application and in the other literature, it is stated that hydrophobic surfaces or membranes must in general be wetted beforehand with water or alcohol, in order to be able to immobilize the nucleic acids with yields that are satisfactory.

On the other hand, for a number of modern applications, such as, for example, the PCR, reversed transcription PCR, SunRise, LCR, branched-DNA, NASBA, or TaqMan technologies and similar real-time quantification methods for PCR, SDA, DNA and RNA chips and arrays for gene expression and mutation analyses, differential display analyses, RFLP, AFLP, cDNA synthesis or substractive hybridization, it is absolutely necessary to be able to release the nucleic acids directly from the solid phase. In this connection, WO 87/06621 teaches that, while the nucleic acids can indeed be recovered from the membranes used in the process, this recovery is fraught with problems and is far from suited to the quantitative isolation of nucleic acids. In addition, the nucleic acid obtained in this manner is, comparatively, extremely diluted, which makes subsequent isolation and concentration steps absolutely necessary.

Summary of the Invention

According to the present invention, all aqueous or other solutions of nucleic acids, as well as all materials and all samples containing nucleic acids, as well as biological samples and materials, foodstuffs, etc. are defined as "nucleic acid samples". In the sense of the present

invention, a sample or a material containing a nucleic acid is defined as a nucleic acid sample and/or a sample preparation which contains the nucleic acids in question. Biological material and/or biological samples in this connection include, e.g., cell-free sample material, plasma, body fluids – such as for example, blood, sputum, urine, feces, sperm, cells, serum, leucocyte fractions, crusta phlogistica, smears; tissue samples of any type, tissue parts and organs; foodstuff samples which contain free or bound nucleic acids or nucleic acid-containing cells; environmental samples which contain free or bound nucleic acids or nucleic acid-containing cells, plants and parts of plants, bacteria, viruses, yeasts and other funghi, other eukaryotes and prokaryotes, etc., as they are published, e.g., in the European patent publication No. EP 743 950 A1, which is incorporated herein by reference, or free nucleic acids as well. In the sense of the present invention, nucleic acids comprise all types of nucleic acids, such as, e.g., ribonucleic acids (RNA) and desoxyribonucleic acids (DNA), in all lengths and configurations, such as double strands, single strand, circular and linear, branched, etc.; monomer nucleotides, oligomers, plasmids, viral and bacterial DNA and RNA, as well as genomic or other non-genomic DNA and RNA from animal and plant cells or other eukaryotes, tRNA, mRNA in processed and non-processed form, hn-RNA, rRNA and cDNA as well as all other nucleic acids that can be envisioned.

For the reasons stated above, the processes known from the state of the art do not constitute - particularly with regard to automation of the process for obtaining nucleic acids - a suitable starting point for an isolation of nucleic acid that is as simple and quantitative as possible from the point of view of process engineering. The purpose of this invention is therefore to overcome the disadvantages of the processes known from the state of the art for the isolation of nucleic acids and to provide a process and method which are capable of being applied or carried out without substantial technical expenditure.

According to the present invention, the aforementioned disadvantages are solved by the processes, isolation and/or reaction devices uses, automatic apparatus kits according to the description, drawings and claims below.

In addition, the invention focuses on processes which make use of surfaces, e.g., porous membranes, on which the nucleic acids can be easily immobilized from the sample containing the nucleic acids, and can again be released by way of similarly easy steps of the process,

whereby the simple performance of the process according to the invention makes it possible to specifically carry out the process in a fully automated manner.

Another purpose of this invention is, in particular, to bind nucleic acids to an immobile solid phase - especially to a membrane - in such a manner that in a subsequent reaction step they can be released immediately from this phase and, if desired, used in other applications, such as, for example, restriction digest, RT, PCR or RT-PCR, as well as any other suitable analytical or enzymatic reaction named above.

Within the scope of the present invention, a surface is defined as any microporous separating layer. This may also directly rest on a substratum and therefore only be accessible from one side or be standing freely in space. Within the meaning of the present invention a membrane is defined as a separating layer which is accessible from both sides when it does not rest with its entire surface area on an impenetrable substratum but is entirely free or is only supported at single points.

Within the meaning of the present invention, isolation is defined as any accumulation of nucleic acids, in which the concentration of nucleic acids is increased and/or the portion of non-nucleic acids in a sample preparation and/or sample is reduced.

The invention provides a process to isolate nucleic acids including the following steps:

- applying at least one nucleic acid sample to a membrane;
- immobilizing the nucleic acids on the membrane;
- releasing the immobilized nucleic acids from the membrane; and
- removing the released nucleic acids through the membrane,

whereby the membrane contains nylon, polysulfone, polyethersulfone, polycarbonate, polyacrylate, acrylic copolymer, polyurethane, polyamide, polyvinylchloride, polyfluorocarbonate, polytetrafluoroethylene, polyvinylidene fluoride, polyethylene-tetrafluoroethylene-copolymerisate, polybenzimidazole, polyethylene-chlorotrifluoroethylene-copolymerisate, polyimide, polyphenylene sulfide, cellulose, cellulose-mix-ester, cellulose-nitrate, cellulose-acetate, polyacrylnitrile, polyacrylnitrile-copolymers, nitrocellulose, polypropylene and/or polyester.

Other membranes also, such as those mentioned below in the present description, may be used for processes according to the invention.

Preferably the loading process takes place from the top and the removal process is carried out in a downward direction; however, flow-through processes, for example, can be envisioned in which a horizontal column is loaded from one side with a solution containing nucleic acid, which, after immobilization of the nucleic acids, penetrates through the membrane and can be removed at the other end of the column.

Preferably, the membrane is situated in a container, e.g., the column mentioned above or any elongated container having an inlet and an outlet, wherein the membrane stretches across the entire diameter of the container.

The membrane may be coated so as to render it hydrophobic or hydrophilic.

Isolation processes to date, especially in isolation columns, function with relatively thick membranes and/or fleeces in order to achieve a complete isolation of the nucleic acids. When the solution is suctioned through the membrane, however, a relatively large, so-called dead-space-volume, i.e., the volume of the membrane, is generated from which the nucleic acids can only be recovered by way of a larger quantity of an elution buffer. This, however, causes the nucleic acids to be more diluted after the elution, which is undesirable or disadvantageous for many applications. For this reason, a preferred embodiment of the invention uses a membrane which is less than 1 mm thick, preferably less than 0.5 mm, and most preferably less than 0.2 mm, e.g., 0.1 mm thick.

The invention furthermore involves a process to isolate nucleic acids with the following steps:

- applying at least one nucleic acid sample to a surface;
- immobilizing the nucleic acids on the surface; and
- releasing the immobilized nucleic acids from the surface with an elution agent.

This process is characterized in that the release takes place at a temperature whose upper limit is 10°C or less and whose lower limit is at the freezing point of the elution agent to be used for such release, so that the elution agent does not freeze. Therefore the following inequation applies: $10°C \geq T \geq T_{S, EM,}$ in which T is the release temperature and $T_{S, EM}$ is the freezing point of the elution agent. We have discovered that, contrary to widespread opinion, a release of the nucleic acids near the freezing point of the elution agent is quite possible. Such an elution at low

temperature even has the unexpected advantage that the nucleic acids are treated more gently and that the activity from any nucleases (DNases or RNases) still present in the sample drops practically to nothing near the freezing point, so that degradation of the nucleic acids is reduced or completely prevented.

Accordingly, the temperature during elution should preferably be even lower, e.g., at less than 5°C. The lower limit may also be at 0°C or -5°C, if the specimen is still liquid at this temperature, based on its ion content. The upper temperature limit should if possible also be low, e.g., at about 5°C.

The process according to the invention therefore requires cooling of the elution buffer and may require cooling of any additional solutions used, as well as cooling of the isolation device if necessary. Since cooling cannot always be guaranteed in a reliable manner, especially during examinations performed in the field, e.g., when screening human samples in developing countries, the present invention also provides an isolation device which allows isolation of nucleic acids at low temperatures independent from any external cooling. For such situations, the instant invention provides an isolation device to isolate nucleic acids having at least an upper part with a top opening, a bottom opening and a membrane, which is located at the bottom opening and which fills the entire diameter of the upper part; a bottom part with an absorbent material; and a collar surrounding the upper part, at least in the area of the membrane, which contains a coolant. The collar containing the coolant allows cooling of the membrane and the solutions placed on the membrane such as the lysate, washing buffer and elution buffer at low temperatures, so that the final elution can take place in a reliable manner within the desired temperature range near the freezing point of the elution buffer.

In an embodiment of this isolation device, the collar has two compartments, which are separated from one another by a mechanically destructible or frangible separation wall, with each of the compartments containing a solution and in which upon mixing of both solutions after destruction of the separating wall, the coolant is generated. The separating wall can be destroyed by the user, e.g., by pressing against the external collar wall, e.g., at points provided for such purpose, and thus causing the separating wall to tear. Suitable solutions to fill the compartments are familiar to practitioners in the area of chemical cooling technology. These may be adjusted to the desired temperatures and to the outside temperatures expected when using the isolation device.

When recovering nucleic acids from biological samples, such as the samples indicated above, it is often necessary to make a lysate the cells or secretions first, in order to be able to reach the nucleic acid. The lysates thus produced may also contain large amounts of undesirable substances in addition to the nucleic acids, such as proteins or fats. If the content of such substances in a lysate is too high, the membrane may become clogged when the lysate is applied, which reduces the efficiency of the nucleic acid isolation and which reduces the permeability of the membrane during washing or elution. In order to avoid this undesirable effect, the invention provides a process in which undesirable substances are removed before they reach the membrane.

In preferred embodiments, the process according to the invention to isolate nucleic acids comprises the following steps:

- adjusting at least one nucleic acid sample to binding conditions which allow immobilization of the nucleic acids contained in at least one of the nucleic acid samples on a surface;
- applying at least one nucleic acid sample to the surface; and
- immobilization of the nucleic acids on the surface,

characterized in that before and/or after adjusting the binding conditions, a pretreatment is applied.

The pre-treatment may, for instance, take place by salting out or by filtration, centrifugation, enzymatic treatment, temperature effect, precipitation and/or extraction of the nucleic acid solution and/or binding contaminants of the nucleic acid solution to surfaces. The pre-treatment may also involve mechanical disruption or homogenizing the nucleic acid solution, if it is for example the lysate of a biological sample.

The binding conditions that were adjusted may permit the immobilization of RNA and/or DNA in this case.

A pre-treatment may be necessary especially in cases when one intends to isolate biological samples with severe contaminants. The biological sample may consist of any conceivable material which is used either immediately or can be recovered from another biological sample. For instance, this may be blood, sputum, urine, feces, sperm, cells, serum, leukocyte fractions, crusta phlogistica, smears, tissue samples, plants, bacteria, funghi, viruses and yeasts, as well as all other types of biological samples mentioned above.

The process according to the invention may be used to its greatest advantage if the biological sample contains a large amount of undesirable substances.

After immobilization of the nucleic acids from the pre-treated nucleic acid sample, the usual isolation steps can be followed, i.e.:

- releasing the immobilized nucleic acids from the surface;
- recovering the nucleic acids released from the surface.

A special advantage of the isolation process according to the invention concerns the fact that it may be connected with chemical reactions, to which the nucleic acids are subjected directly on the surface. A variety of analytical techniques for nucleic acids may therefore be used with the nucleic acids isolated on the surface. In this case it is possible to again release the nucleic acids from the surface prior to the reaction in order to guarantee their free accessibility. Alternatively, a suitable reaction may also be performed with the nucleic acids which are directly bound on the surface.

Accordingly, one aspect of the invention involves a process with a pre-treatment, as outlined above, which is characterized in that the following step preferably takes place at least once after the release stage:

- performing at least one chemical reaction with the nucleic acids.

A special advantage of this process lies in the fact that prior to the chemical reaction, no loss resulting from transfer of the nucleic acids from the isolation device to a reaction device occurs, because the isolation and chemical reaction can take place in the same device.

In an additional aspect not related to pre-treatment, the invention involves a process to carry out a nucleic acid amplification reaction with the following steps:

- applying at least one nucleic acid sample to a surface;
- immobilizing the nucleic acids on the surface; and
- performing an amplification reaction with the nucleic acids.

Especially with the small quantities of material commonly used in amplification reactions or available for use in amplification reactions, it is generally advantageous if the whole reaction sample of nucleic acids can be used in the reaction without any loss from transfer. This is especially advantageous for an automated process since all steps can be carried out in one device. Furthermore, the amount of waste is reduced and the process is faster and more cost-effective.

The amplification reaction may be an isothermal or a non-isothermal reaction.

The amplification reaction may, e.g., consist of an SDA-reaction ("strand displacement amplification"), a PCR, RT-PCR, LCR or a TMA or a rolling circle amplification.

A NASBA-reaction is also possible with this process according to the invention.

Prior to carrying out the amplification reaction, the nucleic acids may be released from the surface with a reaction buffer, whereby the eluate is located on or in the membrane. Alternatively, the amplification reaction may be carried out in a reaction buffer that does not produce a release of the nucleic acids from the surface.

This process preferably produces these additional steps:

- if necessary, release of the reaction products from the surface (to the extent these were still bound during the reaction); and
- removal of the released reaction products from the surface.

Another aspect involves a process to perform chemical reactions with nucleic acids by way of the following steps:

- applying at least one nucleic acid sample to a surface;
- immobilizing nucleic acids on the surface;
- releasing the immobilized nucleic acids from the surface;
- performing at least one chemical reaction with the nucleic acids; and
- removal of the nucleic acids from the surface without prior immobilization.

In this process the nucleic acids are no longer bound (immobilized) to the membrane after the chemical reaction, but removed without binding. Although the eliminationof such an additional step may compromise the purity of the removed specimen, it may be preferred because it saves time in critical applications and it also simplifies certain application methods. A wide range of chemical reactions is available as a result of the process according to the invention. Within the meaning of the invention "chemical reaction" should be defined in this connection as any interaction of the nucleic acids with other substances (with the exception of the surface, since this "reaction" occurs in all processes described herein), i.e., enzymatic modifications, hybridization with probes, chemical sequencing reactions, pH-value-changes, e.g., for basic depurination of RNA and acid depurination of DNA, as well as antibody binding and protein

binding. Generally, each reaction, whether it concerns the changing of covalent bonds or hydrogen bonds, is included.

One advantage of the process according to the invention is the permanent, spatial combination of a volume chamber, in which a great variety of processes can take place, and a membrane to which nucleic acids can be bound. Simply put, this combination allows the manipulation of nucleic acids followed by binding to a membrane. This is especially advantageous for automated processes. After binding to the membrane, the nucleic acids are available for additional treatment steps, e.g., as mentioned above, for isolation of highly pure nucleic acids or for performing chemical reactions with the nucleic acids. An additional aspect of the invention makes it also possible to immediately subject the nucleic acids still bound to the membrane to further analysis, in order to determine certain properties of the nucleic acids.

For that reason the invention also involves a process to analyze nucleic acids in an isolation device with the following steps:

- making available an isolation device with a membrane located therein;
- applying at least one nucleic acid sample to the isolation device;
- immobilizing the nucleic acids on the membrane;
- leading the fluid components of the sample through the membrane; and
- analyzing at least one property of the nucleic acids on the membrane located in the isolation device.

After passing the fluid components through the membrane, at least one chemical reaction as mentioned above can be performed with the nucleic acids in an additional embodiment. This may serve, e.g., to allow the subsequent analysis of the nucleic acids. Examples of reactions in this context are the hybridization of probes, the radioactive labeling of nucleic acids bound to the membrane or the binding of specific antibodies. Auxiliary reactions such as staining nucleic acids, e.g., with intercalating substances such as ethidium bromide should also be considered as a chemical reaction.

Various properties of nucleic acids are open to an analysis while they are bound to the membrane. They have already been described for conventional membranes without a combined reaction device. Some of the properties that can be analyzed are the radioactivity of nucleic acids or their binding affinity for molecules, in which the molecules for example may be antibodies or dye molecules that bind nucleic acids or are bound to nucleic acids or proteins.

This process represents a considerable simplification of the analysis of nucleic acids, since the manipulation of the free membrane is no longer necessary. This is now located in the isolation device.

An irreversible bond of the nucleic acids to the membrane, e.g., for subsequent analytical steps is also within the scope of the present invention. This long-lasting or irreversible bond permits the manipulation of the membrane and the nucleic acids bound thereon to an extent that is not possible for reversibly bound nucleic acids.

An additional aspect of the invention focuses on the quantitative precipitation of nucleic acids.

In previously known methods based on anion-exchange chromatography for purification of 100 μg and more plasmid-DNA (hereinafter indicated as "large scale" DNA purification), the plasmid-DNA is eluted in a high saline buffer from the column during the last step. In order to separate the plasmid-DNA from the salt on the one hand, and to concentrate it on the other, it is precipitated with the aid of alcohol (e.g., isopropanol) and centrifuged in a suitable device. The centrifugation pellet thus obtained is washed with 70% ethanol, in order to remove the residual traces of salt and is then again subjected to centrifugation. The pellet from the second centrifugation is typically dissolved in a small amount of low saline buffer and the plasmid-DNA is processed further in this form.

In addition, the state of the art has proposed processes in which DNA is added in such a form by adding chaotropic salts to the high saline buffer so as to cause binding to silica membranes. After a corresponding washing step, the DNA can again be released from the membrane by way of a low saline buffer.

A similar application is described in a publication (Ruppert et al., *Analytical Biochemistry*, 230: 130-134 (1995)) in which on a small scale (isolation of less than 100 μg of plasmid-DNA) DNA precipitated with isopropanol is bound to PVDF-membranes with pore sizes of less than 0.2 μm, subsequently washed with ethanol and then eluted with TE (Tris-EDTA). However, there is no description of such a method for the large scale process.

The DNA precipitation described with subsequent centrifugation is extremely time-consuming (approx. 1 hour), and furthermore requires the use of centrifuges. In addition to the time factor for this procedure, the last step described for plasmid preparation is particularly prone

to errors. A partial or complete loss of the DNA-pellet also occurs occasionally. A decisive roll appears to be the type (material) of the centrifugation device used.

The use of chaotropic salts (also described) and the subsequent binding of nucleic acids to silica membranes is also time-consuming; moreover, because of the introduction of chaotropic salts to the preparation there is the risk of contamination of the finally isolated DNA.

The filtration of alcoholic precipitates on a small scale as described above has the disadvantage that the operation cannot be transferred linearly to a large scale process. Conventional membranes only permit the isolation of small amounts of nucleic acids, as the membranes are quickly saturated with nucleic acids and no longer absorb anything. When the precipitate buffer is removed and washed, a large portion of the nucleic acids is frequently lost again. In order to avoid this loss, the invention also involves a process to precipitate nucleic acids by way of the following steps:

- making available an isolation device with at least one membrane situated therein;
- applying a nucleic acid sample to the isolation device;
- precipitation of the nucleic acids contained in the sample with alcohol, so that the nucleic acids are at least bound to a membrane. The process is characterized in that the pore size of at least one membrane is the same or greater than 0.2 micrometers.

Alcohols considered to perform the process according to the invention are first of all hydroxyl derivates of aliphatic or acyclical saturated or unsaturated hydrocarbons.

Among the aforementioned hydroxyl compounds, the C_1-C_5 alkanols, such as methanol, ethanol, n-propanol, n-butanol, tert.–butanol, n-pentanol or mixtures thereof are preferred. Especially preferred is the use of isopropanol to carry out the process according to the invention.

In this process, the alcohol can be mixed with this solution before or after loading the isolation device with the solution containing the nucleic acid. The volume ratio of the nucleic acid-containing solution to alcohol, especially isopropanol, preferably is 2:1 to 1:1, most preferably 1.67:1 to 1:1, and for example 1.43:1.

The surface of the membrane is preferably chosen so that all the nucleic acids contained in the solution can be bound to the membrane.

The invention also involves the use of membranes with a pore size of equal or larger than 0.2 μm to bind the alcohol-precipitated nucleic acids, which may consist of DNA and/or RNA.

Especially advantageous is the use of a 0.45 µm cellulose acetate or cellulose nitrate filter and/or the use of various layers of a 0.65 µm cellulose acetate or cellulose nitrate filter. The procedure can both be used as vacuum filtration and as pressure filtration.

The process according to the invention permits a time-saving transfer of nucleic acids from a high-salt buffer system to a low-salt buffer system, which is possible without use of complex apparatus. It is suitable as a substitute for the classical alcoholic precipitation of DNA from a high-salt buffer, which is typically by centrifugation steps. Because of the great effectiveness of the method (minor loss of yield) it is especially suitable as a preparation for a large scale process. Furthermore the process according to the invention does not introduce any additional substances in the already purified nucleic acids. In addition, compared to the classical method, susceptibility to errors is less (loss of the centrifugation sediment during the washing cycle is not possible using the process of the invention).

Preferably, applying the solution should take place from the top in the various processes explained above. In principle, a wide range of methods are available which pass various solutions such as nucleic acid-containing immobilization buffers, washing buffers, eluate, etc. through the membranes.

This may be achieved through gravity, centrifugation, vacuum, positive pressure (on the loading side), and capillary forces.

Between the immobilization and the separation step, the immobilized nucleic acids may be washed with at least one washing buffer. The washing preferably consists of the following steps for each washing buffer:

- applying a predetermined quantity of washing buffer to the surface, and
- passing the washing buffer through the surface.

The application and immobilization of the nucleic acids may again consist of the following steps:

- mixing of the nucleic acid sample with an immobilization buffer;
- applying the nucleic acid sample with the immobilization buffer on the surface, and
- passing the liquid components through the surface in essentially the direction of the loading step.

The processes have the major advantage that they can easily be automated, so that at least one of the steps can be fully automated in an automatic device. It is also possible to have all steps of the processes performed in a pre-arranged sequence by an automatic apparatus. Especially in these cases, but also for manual handling, it is possible that a majority of nucleic acids are simultaneously subject to isolation. For example, multi-isolation devices may be used in the form of commonly available "multi-well" devices with 8, 12, 24, 48, 96 or more single isolation wells.

The removal of the nucleic acids may take place in two roughly different directions. On the one hand it is possible to feed (pass) the (eluted) nucleic acids that were removed through the membrane and to remove them toward the membrane's side, that is located opposite the side on which the nucleic acid-containing solution and/or the lysate was placed. In this case the nucleic acid is removed in the direction of its passing through the membrane. The other possibility consists of removing the nucleic acids from the membrane and/or from the surface on the side where they were introduced. The removal then takes place in the direction opposite to their introduction or "in the same direction", in which they were introduced; in other words, on the side where they were introduced. In this case the nucleic acids do not pass through the membrane. In some of the processes according to the invention, removal of the nucleic acids takes place through the membrane in the direction they were introduced. In the event a process is carried out with a surface that does not have a non-permeable substratum, e.g., a synthetic layer, the removal can of course only take place in the direction of introduction (hence in the opposite direction). For a few processes, however, the substance can be removed in both directions.

If the nucleic acids are eluted (released) from the surface essentially in the opposite direction from the direction in which they were introduced and immobilized, "the same direction" is essentially considered each direction with an angle equal or smaller than 180°, compared to the direction of introduction, so that upon elution, the nucleic acids under no circumstances permeate the surface, e.g., a membrane, but are removed from the surface in the direction opposite from the loading direction in which they were introduced to the surface. In preferred embodiments, on the other hand, the other buffers, i.e., those buffers which contain nucleic acids during the loading process, and if required a washing buffer, are suctioned through the surface or otherwise transferred. If the isolation takes place on a membrane located in a

device, whereby the membrane fills the entire diameter of the device, the preferred loading method is from the top. In this case the removal step again occurs upward. Figure 2 shows an example of a funnel-shaped isolation device, which is loaded from the top and in which the removal of the nucleic acids takes place in an upward direction.

It is understood that, in the case of removal in a direction opposite to introduction, other configurations are also imaginable, e.g., removal of the nucleic acids from below. It is possible, for example, to suction a buffer containing nucleic acids, such as a lysate buffer from a reaction device directly into an isolation device by way of a suction installation, so that the nucleic acids will be bound to the bottom of a membrane in the isolation device. In such a case, the removal of the nucleic acids from the surface can be carried out, in such a way that an elution buffer is suctioned up from below and is drained again downward into a device after separation of the nucleic acids. The removal of the nucleic acids therefore also takes place in a downward direction.

A lateral removal of the nucleic acids is also possible, e.g., if a horizontal column with a membrane located therein is loaded with a lysate during the flow-through process and the horizontal column is subsequently washed with elution buffers on the side of the membrane to which the nucleic acids are bound.

An example for the maximum possible angle of 180° is a slope with a surface suitable to bind nucleic acids along which surface the various solutions and/or buffers flow. Like all buffers, the elution buffer also arrives from one side and is drained on the other side. In this case, the inflow direction of the buffer and the draining direction of the buffer with the nucleic acids included therein make an angle of 180°; the removal, however, continues to take place on the same side of the surface as the immobilization.

Following the process according to the invention, the sample containing nucleic acids described above is added to a solution which contains the appropriate salts and/or alcohol(s); subsequently the sample is lysed, if necessary, and the mixture obtained in this manner is led by way of a vacuum, centrifugation, positive pressure, capillary forces or by way of other appropriate processes, through a porous surface, whereby the nucleic acids are immobilized on the surface.

Suitable salts for the immobilization of nucleic acids on membranes or other surfaces and/or for the lysis of nucleic acid samples are salts of metal cations, such as alkaline or alkaline earth metals, with mineral acids; especially alkaline or alkaline-earth halides and/or sulfates or phosphates, including the halides of sodium, lithium or potassium or magnesium sulfate, which are most preferable. Other metal cations, e.g., Mn, Cu, Cs or Al, or the ammonium cation can be used, preferably as salts of mineral acids.

Furthermore to carry out the process according to the invention, salts having one or more basic functions or even polyfunctional organic acids with alkaline or alkaline-earth metals are suitable. These especially include sodium, potassium or magnesium salts with organic dicarboxylic acids, such as e.g., oxalic, malonic or succinic acids, or with hydroxy and/or polyhydroxycarboxylic acids, such as, e.g., with citric acids, preferably.

The substances indicated above to immobilize the nucleic acids on surfaces and/or for the lysis of nucleic acid samples may be used separately or in mixtures, if this should prove to be more suitable for certain applications.

In this connection the use of so-called chaotropic agents has proved to be particularly effective. Chaotropic substances are able to disrupt the three-dimensional structure of hydrogen bonds. This also weakens the intramolecular binding forces which are involved in the formation of spatial structures, such as, e.g., primary, secondary, tertiary or quaternary structures, in biological molecules. Suitable chaotropic agents are well known to those skilled in the art (see, Römpp, Lexikon der Biotechnologie, Publisher H. Dellweg, R.D. Schmid and W.E. Fromm, Thieme Verlag, Stuttgart 1992).

According to this invention preferred chaotropic substances are salts from the group of trichloroacetates, thiocyanates, perchlorates, iodides or guanidinium hydrochloride and urea. The chaotropic substances are then used in a 0.01 to 10 molar aqueous solution, preferably in a 0.1 to 7 molar aqueous solution, and most preferably in a 0.2 to 5 molar aqueous solution. In this connection the aforementioned chaotropic agents can be used individually or in combination. Most preferably 0.01 to 10 molar aqueous solutions, or 0.1 to 7 molar aqueous solutions, or 0.2 to 5 molar aqueous solutions of sodium perchlorate, guanidinium hydrochloride, guanidinium isothiocyanate, sodium iodide and/or potassium iodide are used.

The salt solutions used in the processes according to the invention for lysis, binding, washing and/or for elution are preferably buffered. All suitable buffer systems can be considered

as buffer substances, such as, e.g., carboxylic acid buffers, especially citrate buffers, acetate buffers, succinate buffers, malonate buffers as well as glycine buffers, morpholino-propane-sulfone-acids (MOPS) or Tris (hydroxymethyl) aminomethane (Tris) in concentrations of 0.001 to 3 mol/liter, preferably 0.005 to 1 mol/liter, and most preferably 0.01 to 0.5 mol/liter, and particularly preferred 0.01 to 0.2 mol/liter.

To carry out the process according to the invention, first all hydroxyl derivates of aliphatic or acyclical saturated or unsaturated hydrocarbons are eligible as alcohols. It is irrelevant whether these compounds contain one, two, three or more hydroxyl groups – such as polyvalent C_1-C_5 alkanols, e.g., ethylene glycol, propylene glycol or glycerin.

In addition, the alcohols that can be used according to the invention also include sugar derivates, the so-called aldites, as well as phenols, e.g., polyphenols.

Among the aforementioned hydroxy compounds, C_1-C_5-alkanols, such as methanol, ethanol, n-propanol, tert.-butanol and pentanols, or mixtures of such alcohols, are most preferred.

Within the meaning of this invention, such substances and/or membranes which by their chemical nature easily mix with water or absorb water are considered hydrophilic.

Within the meaning of this invention, such substances and/or membranes which by their chemical nature do not penetrate water or vice-versa and which cannot stay dissolved in water are considered hydrophobic.

Within the meaning of this invention, any microporous separating layer is understood to be a surface. In the case of a membrane the surface consists of a film made of polymer material. The polymer preferably consists of monomers with polar groups.

In a further embodiment of the process according to the invention, the concept of surface furthermore also comprises a layer of particles and/or a granulate as well as fibers such as silica gel fleece.

When hydrophobic membranes are used in the practice of this invention, membranes are preferred which consist of a hydrophilic basic material and which are made hydrophobic by a corresponding chemical post-treatment which is known from the state of the art. Membranes such as commercially available hydrophobic nylon membranes are preferably used.

According to the invention membranes that are hydrophobic are generally defined as those membranes which are originally hydrophilic membranes that have been coated with hydrophobical coating agents mentioned below. Such hydrophobical coating agents coat the

hydrophilic substances with a thin film of hydrophobic groups, which, e.g., include longer alkyl chains or siloxane groups. Many suitable hydrophobic coating agents are known and include, e.g., paraffins, waxes, metallic soaps, etc., if necessary with additions of aluminum, zirconium salts, quaternary organic compounds, ureic derivates, lipid-modified melamine resins, silicones, zinc organic compounds, glutaric dialdehydes, and similar compounds.

According to the invention suitable hydrophobic membranes also are those membranes which are by themselves hydrophobic or which have been made hydrophobic and whose basic material may contain polar groups. According to these criteria, e.g., especially hydrophobic materials from the following group are suitable for use according to the invention: Nylon, polysulfones, polyether sulfones, cellulose nitrate, polypropylene, polycarbonates, polyacrylates as well as acrylic copolymers, polyurethanes, polyamides, polyvinyl- chloride, polyfluorocarbonates, polytetrafluoroethylene, polyvinylidene fluoride, polyethylene-tetra-fluoroethylene copolymerisates, polyethylene-chlorotrifluoro-ethylene-copolymerisates, or polyphenylene sulfide, as well as cellulose and cellulose-mix esters, cellulose acetate or nitrocellulose as well as polybenzimidazoles, polyimides, polyacryl nitriles, polyacrylnitril-copolymers, hydrophobisized glass fiber membranes, including hydrophobisized nylon membranes which are most preferable.

Preferred hydrophilic surfaces include hydrophilic materials *per se* and also hydrophobic materials which have been hydrophilisized. For instance the following substances can be used: hydrophilic nylon, hydrophilic polyether-sulfones, hydrophilic polycarbonates, hydrophilic polyesters, hydrophilic poly-tetra-fluoroethylenes on polypropylene tissues, hydrophilic polytetrafluoroethylenes on polypropylene fleece, hydrophilisized polyvinylidene fluoride, hydrophilisized polytetrafluoroethylenes, hydrophilic polyamides, nitrocellulose, hydrophilic polybenzimidazoles, hydrophilic polyimides, hydrophilic polyacryl-nitriles, hydrophilic polyacrylnitril-copolymers, hydrophilic polypropylene, cellulose nitrate, cellulose-mix-ester and cellulose acetate.

The membranes described above are already known in the art, partially for their use in nucleic acid binding, but not yet in the context of the invention. A series of materials for this particular use is, however, not known from the state of the art. The extensive trials disclosed herein have demonstrated that there are additional membranes that are suitable to bind nucleic acids.

The present invention therefore also involves the use of cellulose acetate, non-carboxylized, hydrophobic polyvinylidene fluoride, or massive hydrophobic poly-tetra-fluorethylene as a material on which to precipitate and isolate nucleic acids. In this context, the term "massive" denotes a material which generally consists of the corresponding compound and is neither coated nor applied as a coating on a carrier material.

The material may be used as a membrane, as granulate, as fibers or in other suitable forms. The fibers may, e.g., be configured as fleece and the granulate may be pressed as a grid.

The membranes used in the process described above according to the invention (with the exception of isopropanol precipitate) for instance have a pore diameter of 0.001 to 50 µm, preferably 0.01 to 20 µm, and most preferably a pore size of 0.05 to 10 µm. In case the nucleic acids are precipitated with isopropanol according to the process described above, the pore size must be greater than 0.2 µm.

The salts or alcohols described above or the phenols or polyphenols may also be considered as washing buffers. Detergents and natural substances in the broadest sense of the word, such as albumin, or milk powder may also be used for the washing steps. The addition of chaotropic substances is also possible. Polymers as well as detergents with dissolving abilities and similar materials may also be added. The washing buffers and the substances contained therein should at any rate generally be able to bind undesirable contaminants, to dissolve them or to react with them, so that these contaminants or their decomposition products can be removed jointly with the washing buffer.

The temperatures during the washing stage typically range from about 10° to 30°C, preferably at room temperature, although higher or lower temperatures may also be applied successfully. When elution is performed at a low temperature, e.g. 2 °C, one should not forget to also cool the washing buffer in order to pre-cool the temperature of the isolation device and the surface and/or membrane to the desired temperature. One application for low temperatures is cytoplasmatic lysis, during which the cell nuclei remain undamaged. Higher temperatures of the washing buffers on the other hand cause better dissolution of the contaminants to be washed out.

Suitable eluting agents for the purposes of the invention are water or aqueous salt solutions. Buffer solutions that are known from the state of the art are used as salt solutions, such as morpholinopropane sulfonic acid (MOPS), tris(hydroxymethyl) aminomethane (TRIS),

2-[4-(2-hydroxyethyl) piperazino]ethane sulfonic acid (HEPES) in a concentration from 0.001 to 0.5 moles/liter, preferably 0.01 to 0.2 moles/liter, most preferably 0.01 to 0.05 molar solutions. Also preferred for use are aqueous solutions of alkaline or alkaline-earth metal salts, in particular, their halogenides, for example, including 0.001 to 0.5 molar (preferably 0.01 to 0.2 molar, most preferably 0.01 to 0.05 molar) aqueous solutions of sodium chloride, lithium chloride, potassium chloride or magnesium chloride. Also preferred for use are solutions of salts of the alkaline or alkaline-earth metals with carboxylic or dicarboxylic acids, e.g., oxalic acid or acetic acid, or solutions of sodium acetate or sodium oxalate in water, e.g., in the concentration range mentioned above, such as 0.001 to 0.5 molar, preferably 0.01 to 0.2 molar, most preferably from 0.01 to 0.05 molar.

The addition of subsidiary compounds such as detergents or DMSO is also possible. If a chemical reaction must be carried out with the eluted nucleic acids, either directly on the membrane or in another reaction device, it is also possible to add such substances or other subsidiary compounds which are to be used in the reaction to the elution buffer. For instance, the addition of DMSO in low concentrations is customary in many reactions.

After a chemical reaction with the nucleic acids, these can also be eluted with the reaction buffer. For instance, the nucleic acids can be eluted with the reaction buffer or the reaction master mix after a SDA- or a NASBA-reaction.

Most specifically, pure water is the preferred elution agent, e.g., demineralized, bi-distilled, or ultra pure millipore water.

The elution can, for example, be carried out successfully at temperatures from below 0°C to 90°C, e.g., from 10° to 30°C or at higher temperatures. It is also possible to elute with water vapor. The lower limit of the elution temperature is, as explained above, the freezing point of the elution buffer.

Based on the smooth executability of the processes according to the invention which can also be performed "in the field", i.e., outside of established laboratory installations and therefore without extensive electrically powered equipment, the invention also involves the preparation of isolation devices with which the process according to the invention can be carried out with a minimum of additional subsidiary materials. For this, a reaction device can be used which contains a membrane. This can be brought into contact with an absorbent material, such as a

sponge, in order to absorb the various buffers used through the membrane. The sponge acts therefore as a combination vacuum pump or centrifuge in conjunction with a waste collector. In order to recover the eluate, contact of the absorbent material with the membrane is eliminated, so that the eluate cannot be lost, but instead can be removed or studied further.

In this aspect, the invention specifically involves an isolation device to isolate nucleic acids with at least a cylindrical upper part with a top opening, a bottom opening and a membrane which is located at the bottom opening and fills the entire diameter of the upper part; is equipped with a bottom part containing an absorbent material; and a mechanism for the connection between the upper and lower parts, in which, after the connection has been made, the membrane is in contact with the absorbent material, and when the connection is not made, the membrane is not in contact with the absorbent material.

Preferably, the bottom or lower part is a cylinder with the same diameter as the upper part. In this manner, a simple tube is obtained having essentially a constant diameter, which can be handled in the same way as traditional reaction devices. Especially if the upper part or the upper part plus lower part create a tube which can be placed in reaction device holders, such as those used in laboratories, this effect can be achieved. The mechanism can be a connection which allows a spatial separation of the upper and lower parts, for example a bayonet socket, a plug-in socket or a threaded end. A bayonet socket has the advantage that it is easier to lock and unlock, whereas the threaded connection allows for a better, more watertight connection of the upper and lower parts. Alternatively, a pre-determined breaking point can be provided between the upper and lower parts, which at least allows for the one-time separation of both parts and which can be manufactured at a very low price. Alternatively, the connection can also be a sliding mechanism which can be slid between the absorbent material and the membrane. In this embodiment, a separation of membrane and absorbent material can be achieved as well.

To increase the processing capacity and to be able to carry out the process according to the invention even more economically, it is also possible to modify the isolation device according to the invention described above in such a way that various upper parts are placed on a bottom part. The bottom part can serve simultaneously as a holder of the assembly and in addition have such dimensions that a variety of isolation processes, at least more than mere connections for the upper and lower parts, are available, and can be carried out before the suction capacity of the absorbent material in the bottom part is exhausted.

The absorbent material in the lower part may contain a sponge and/or a granulate. The granulate can consist of a superabsorbent material, as is known by those skilled in the art of absorption technology (e.g., for hygiene-related items).

The invention similarly involves utilization of this isolation device according to the invention for the analysis of properties of nucleic acids and to isolate nucleic acids.

With respect to the separate stages, the processes according to the invention are typically carried out as follows:

When starting from biological samples, they must first be subjected to lysis in the appropriate buffers. Additional processes to achieve lysis may be needed, e.g., a mechanical action, such as homogenization or ultrasound, enzymatic reaction, temperature changes or additives. In case it is required or desirable, a pre-treatment can follow this lysis in order to remove debris from the lysate. Subsequently, in case this has not happened yet, the conditions in the lysate are adjusted, so that immobilization of the nucleic acids on the surface can take place. Even after adjustment of the binding conditions, a pre-treatment step can follow cumulatively or alternatively to the above pre-treatment step .

This pretreated lysate of the sample used for the recovery of nucleic acids or the originally free nucleic acid(s) –if one did not start from a biological sample– is/are pipetted, for example, in a (plastic) column, in which the hydrophobic membrane is fastened, for example, on the floor. It is more efficient if the membrane is fastened to a grid, which serves as a mechanical support. The lysate is then conducted through the membrane, which can be achieved by applying a vacuum at the outlet of the column. The transport can also be accomplished by applying positive pressure to the lysate. In addition, as mentioned above, the transport of the lysate can take place by centrifugation or by the effect of capillary forces. The latter can be produced, for example, with a sponge-like material which is introduced below the membrane and is in contact with the lysate or filtrate. In the case of centrifugation, the isolation device open at the bottom may be used in a collection tube for the flowthrough liquid.

The washing stage included in the preferred embodiments can take place if the washing buffer is transported through the surface of the membrane or is remaining on the same side of the surface as the nucleic acids. If the washing buffer is transported or suctioned through, this can take place in different ways, e.g., by a sponge located on the other side of the membrane, a suction or positive pressure mechanism or by centrifugation or gravity.

The advantage of a configuration utilizing an absorbent, possibly spongy material is that it provides a simple, secure and handy means for disposing of the filtrate, in this case only the sponge, which by that time is more or less saturated with the filtrate and needs to be replaced. At this point it is clear that the column can be operated continuously or also in a batch-like manner, and that both modes of operation can be fully automated, until the membrane is saturated with nucleic acids. In the last stage, if required, the elution of the nucleic acids takes place, which for example can be pipetted or lifted from the membrane or can be removed upward in another way, if no *in situ* analysis of the nucleic acids that are still bound is to be performed.

The desired nucleic acids are present in very small volumes of buffers with no or low salt concentrations, which is a great advantage for all molecular biological analyses, since it is always desirable to have pure nucleic acids in high concentrations and in the smallest volumes possible. In order to obtain the smallest possible volumes of eluate, it is especially preferred to use as surfaces those membranes that are as thin as possible, so that only very little liquid can accumulate in them.

Furthermore, the present invention offers the advantage that in the case of a vertical configuration of the device (where the membrane is placed in a horizontal direction) the volume located above the membrane can be used as a reaction chamber. Hence, it is possible, for example, after isolation and removal of the nucleic acids recovered according to the process of the invention, to not remove them immediately but to leave them in the isolation device and to subject them to a molecular biological application, such as restriction digest, RT, PCR, RT-PCR, *in vitro* transcription, NASBA, rolling circle, LCR (ligase chain reaction), SDA (strand displacement amplification) or enzyme reactions, such as RNase- and DNase-digestion for the complete removal of any of the nucleic acids that are not wanted, to bind the nucleic acids resulting from these reactions again to the membrane according to the process according to the invention or to leave them in the supernatant, if necessary to wash them as described, and subsequently to elute them, to isolate and/or analyze them, e.g., by way of chromatography, spectroscopy, fluorometry, electrophoresis, or similar measurements.

The nucleic acids isolated according to the invention are free of enzymes that degrade the nucleic acids and have such a high purity that they can immediately be used and processed in the greatest variety of ways.

The nucleic acids produced according to the invention can be used for cloning and as substrates for a great variety of enzymes, such as, e.g., DNA-polymerases, RNA-polymerases such as, e.g., T7-polymerase or T3-polymerase, DNA-restriction enzymes, DNA-ligase, reverse transcriptase and others.

The nucleic acids produced by the processes of the invention are especially suitable for amplification, especially for PCR, strand displacement amplification, rolling circle processes, ligase chain reaction (LCR), SunRise, NASBA and similar processes.

The processes according to the invention are furthermore extremely suitable to produce nucleic acids for their use in diagnostics, e.g., in food analysis, in toxicological examinations, in medical and clinical diagnostics, in diagnostics of germs, gene expression analysis, and in environmental analysis. The processes are especially suitable for a diagnostic process, which is characterized in that the nucleic acids purified by way of the processes according to the invention are amplified in a subsequent step, and the nucleic acids that are thus amplified are detected subsequently and/or simultaneously (see, e.g., Holland et al., 1991, *Proc. Natl. Acad. Sci.*, 88: 7276 – 7280; Livak et al., 1995, *PCR Methods Applic.*, 4: 357 –362; Kievits et al., 1991, *J. Virol. Meth.*, 35: 273 – 286; Uyttendaele et al., 1994, *J. Appl. Bacteriol.*, 77: 694 – 701).

Moreover, the processes according to the invention are especially suitable to produce nucleic acids which, in a subsequent step, are subjected to a signal amplification step based on a hybridization reaction, which is specifically characterized by the fact that the nucleic acids produced in the process according to the invention are brought into contact with "branched nucleic acids", especially branched DNA and/or branched RNA and/or corresponding dendritic nucleic acids and the signal that is generated is detected, as described in the following literature (e.g., Bresters et al., 1994, *J. Med. Virol.*, 43(3): 262 – 286; Collins et al., 1997, *Nucl. Acids Res.*, 25(15): 2979 – 2984).

An example of automation of a process according to the invention is explained below and examples to perform the process with different surfaces and nucleic acids are also described. In this description reference is made to the attached figures which illustrate the following.

Brief Description of the Drawings

Figure 1 shows automatic equipment suitable to perform the process according to the invention in a stylized graph.

Figure 2 shows a first embodiment of an isolation device and collector to perform the process according to the invention.

Figure 3 shows a second embodiment of an isolation device and collector to perform the process according to the invention.

Figure 4 shows a third embodiment of an isolation device and collector to perform the process according to the invention.

Figure 5 shows embodiments of isolation devices with an upper part according to the invention.

Figure 6 shows the ethidium bromide stained gel of an electrophoretic separation of various samples according to the process of the invention.

Figure 7 shows another ethidium bromide stained gel of an electrophoretic separation of various samples according to the process of the invention.

Figure 8 shows another ethidium bromide stained gel of an electrophoretic separation of various samples according to the process of the invention.

Figure 9 shows the ethidium bromide stained gel of an electrophoretic separation of various samples according to the process of the invention.

Figure 10 shows another ethidium bromide stained gel of an electrophoretic separation of various samples according to the process of the invention.

Figure 11 shows another ethidium bromide stained gel of an electrophoretic separation of various samples according to the process of the invention.

Description of the Preferred Embodiments

The processes according to the invention are preferably performed in an automatic manner either partially or completely, in other words, in all stages. An example for suitable automatic equipment is illustrated in Figure 1, in which a main part 1 is equipped with control electronics and driving engines with a work platform 3 and a movable arm 2. Various elements are positioned on the work platform, such as area 4 to hold various devices. A vacuum manifold 5 serves to absorb liquids from isolation devices which are placed above it and are open at the bottom, or otherwise with the devices connected to the vacuum manifold. A shaker 6 is also provided, which can be used, e.g., for the lysis of biological samples. The isolation device assemblies used are, e.g., injection-molded parts with integrated isolation devices, in which the surfaces according to the invention are included. Typically 8, 12, 24, 48, 96 or up to 1536

isolation devices can be used as these are available for example in the formats of modern multi-well-plates. Even higher numbers of isolation devices might be possible in one plate, if standards are available. With the aid of Luer-adapters it is, however, also possible to make separate bottoms of the assembly available and to equip these with one or more isolation devices as needed. Isolation devices used individually without Luer-adapters are also included in the invention.

Under a vacuum and dispensing mechanism 8 the isolation devices are placed in the automatic apparatus and with these, liquids can be taken up and dispensed. In this assembly several single vacuum units may be provided, so as to make the simultaneous processing of an isolation or reaction device possible. The vacuum and dispensing mechanism 8 therefore acts as a pipet. Vacuum and pressure are fed to the vacuum and dispensing mechanism 8 via tube 9.

To isolate the nucleic acids, reaction devices with cells may for example be placed in the shaker/holder 6, into which lysis buffers are introduced with the help of the dispensing mechanism. After mixing, the cell lysates are transferred to isolation devices. The lysis buffer is subsequently passed through the surfaces in the isolation devices. Subsequently, the surfaces may be washed with a washing buffer in order to remove cell lysate residues, in which also the washing buffer is drained off downward. Finally, an elution buffer is dispensed into the isolation devices and after repeated shaking the separated nucleic acids are removed from above and transferred to collection microtubes.

Usually, disposable tips are used on the vacuum and dispensing mechanism 8 to prevent contamination of the samples.

Figures 2 through 4 show different schematic examples for suitable isolation devices to be used according to the present invention.

In Figure 2, a funnel-shaped isolation device 10 is provided with a surface 11, e.g., a membrane, which is placed on a collector 12, which contains a sponge-like material 13 that serves to absorb the lysis and washing buffers. Under the sponge-like material 13 a superabsorbent layer 14 may be placed to improve the suction performance. Alternatively, layer 14 may also contain a material which is chemically able to react with water, e.g., acrylate. The water is therefore also removed from the process. Lysate or another preparation of nucleic acids is placed in the funnel. The sponge-like material 13 absorbs the applied liquid through membrane 11. Prior to the addition of the elution buffer, the sponge is moved some distance

from the membrane, e.g., by a mechanism inside a collector 12 (not visible in the drawing). This will prevent the elution buffer in the last stage from being also suctioned through membrane 11. This buffer, however, stays on the surface (Figure 2b) and can be removed together with the nucleic acids from above. When using this assembly, the vacuum mechanism 5 in the automatic apparatus is no longer necessary.

Figure 3 shows another example of an isolation device, which is connected to a collector 16 via a Luer-connection located at the bottom via a Luer-adapter 17, which in this case does not contain a sponge, but is connected to a vacuum mechanism via a muff 18. Lysis and washing buffers may in this case be suctioned through membrane 11 by creating a vacuum (Fig. 3a). When the eluate buffer is introduced, the vacuum remains turned off, so that the eluate can be removed from above (Fig. 3b). With the use of a Luer-connection, individual isolation devices can be removed from the isolation device assembly. It will be understood, however, that the vacuum collector can also be combined with fixed isolation devices, e.g., multi-well devices containing 8, 12, 24, 48, 96 or more single devices.

Figure 4 finally shows an embodiment which provides a collector, into which the buffers are suctioned through the membrane or surface 11 by way of gravity or centrifuged. The eluate buffer, which is used in small volumes, is not heavy enough itself to penetrate membrane 11 and can again be removed from above (Fig. 4b).

Figure 5 shows embodiments of the isolation devices according to the invention.

In Figure 5A, an isolation device with a cylindrical upper part 20 has been illustrated. This upper part is connected to a bottom part 22 by way of a threaded connection 25. Instead of the threaded connection other types of connections may also be used, to the extent these permit a watertight connection of the upper and bottom parts and provide a possibility of introducing membrane 11. In this embodiment, membrane 11 is applied directly to the bottom opening of upper part 20. It may, however, also be moved inward or be placed at an angle other than 90° with respect to the upper part's wall. The bottom part also has a cylindrical shape, but may be of a different design in other embodiments. For example, a quadrangular shape may be used, which improves the stability of the upper part 20 on a surface. The widening of bottom part 22 compared to upper part 20 is also possible, for example in case a larger cavity is required in

bottom part 22 in certain embodiments of the process according to the invention in order to fully absorb the solutions used in the absorbent material 13.

An alternative embodiment to the embodiment shown in Figure 5A is illustrated in Figure 5B. In this case upper part 20 and bottom part 22 are fixed to one another or may also be built in one piece. Between the absorbing material 13 and membrane 11, a sliding mechanism 27 may be slid via an opening 26 into the isolation device to separate membrane 11 and absorbent material 13 from one another. In this example sliding mechanism 27 is equipped with an additional handle 28, which facilitates pulling out sliding mechanism 27. The sliding mechanism can, however, also be designed without this handle. As shown in Figure 5B, the absorbent material 13 expands slightly, to be able to bridge the space taken up by the sliding mechanism and to make contact with the membrane.

Figure 5C shows another embodiment of the isolation device according to the invention. In this case the bottom part 23 is equipped with several connections 30 to accommodate the upper parts 20, thus permitting the simultaneous processing of a multiplicity of samples. The upper parts 20 in this example are connected with bottom part 23 by way of threaded connections 31. Although shown smaller in the illustration than the upper parts 20 of Figures 5A and 5B, it is understood that the upper parts can be the same size (or can be larger or smaller) as indicated in those embodiments.

Finally, Figure 5 D shows an isolation device according to the invention with a collar 32 with coolant, which surrounds membrane 11 on the outside. In this embodiment, upper part 20 and bottom part 24 are connected to one another by way of a plug-in socket. Another type of connection or a one-piece version are, however, also possible. Collar 32 consists of two compartments, 33 and 34, which can be connected with one another by destroying the separating wall 35. Both compartments 33, 34 are loaded with substances, e.g. solutions, which, when mixed after destruction of the separating wall 35, causes the temperature of the entire mixture to drop.

The invention described above will be further explained in the following examples. Different and alternative designs of the devices and processes will become clear to the skilled practitioner from the description above and from the following examples. It should expressly be pointed out, however, that these examples and the description accompanying these examples

only serve as an illustration of the invention and are not to be considered a limitation of the invention.

Example 1: Isolation of total RNA from HeLa cells

Commercially available nylon membranes (for example, a material from MSI, "Magna SH" with a pore diameter of 1.2µm, or a material from Pall GmbH, "Hydrolon" with a pore diameter of 1.2 µm), which are chemically post-treated and to be hydrophobic, were placed as a single layer in a plastic column. The membranes were placed on a polypropylene grid which served as a mechanical support. The membranes were fixed in the plastic column with a ring. The column prepared in this manner was connected by means of a Luer connection to a vacuum chamber. All the isolation steps were carried out through the application of a vacuum.

For the isolation, 5 x 10^5 HeLa cells were harvested by centrifugation and the supernatant removed. The cells were lysed by the addition of 150 µl of a commercial guanidium isothiocyanate buffer (e.g., RLT buffer from QIAGEN GmbH, Hilden, DE), in a manner thoroughly familiar to those skilled in the art. Lysis was promoted by roughly mixing by pipetting or vortexing for 5 seconds. Then 150 µl of 70% ethanol were added and mixed in by repeatedly pipetting or by vortexing for about 5 seconds.

The lysate was transferred into the plastic column and suctioned through the membrane by evacuating the vacuum chamber. Under these conditions, the RNA remained bound to the membrane. Next, washing was performed using a first commercial washing buffer containing guanidium isothiocyanate (e.g., with RW1 buffer from QIAGEN GmbH) and, after that, with a second washing buffer containing TRIS or TRIS and alcohol (e.g., with the RPE buffer from QIAGEN GmbH). The washing buffers in each case were suctioned through the membrane by evacuation of the vacuum chamber. After the final washing step, the vacuum was maintained for a period of about 10 minutes, in order to dry the membrane, after which the vacuum was switched off.

For the elution, 70 µl RNase-free water was pipetted onto the membrane in order to dissolve the purified RNA from the membrane. After incubation for one minute at a temperature in the range from 10° to 30°C, the eluate was pipetted from the membrane from above and the elution step was repeated in order to make sure that the elution was complete.

The quantity of isolated total RNA obtained in this manner was determined by spectrophotometric measurement of the light absorption at 260 nm. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

The results of the two isolations with hydrophobic nylon membranes (Nos. 1 and 2) are shown in Table 1, compared with experiments in which on the one hand a hydrophilic nylon membrane (Nyaflo) (No. 3) and a silica membrane (No. 4) were used. The values reported in the table provide convincing support for the impressive isolation yield and separation effect of the materials used in accordance with this invention. They also show that silica gel-fleece clearly produces a lower yield, which presumably can be attributed to its fleecelike structure and the ensuing absorption of a large portion of the eluate buffer.

Table 1: RNA yield and purity of total RNA isolated according to Example 1.

Sample No.	Type of Membrane	Yield of Total-RNA (µg)	Absorbance E_{260}/E_{280}
1	Magna SH 1.2 µm (hydrophobic nylon)	6.0	1.97
2	Hydrolon 1.2 µm (hydrophobic nylon)	7.1	2.05
3	Nylaflo (hydrophilic nylon)	< 0.2	Not Determined
4	hydrophilic silica membrane	< 0.2	Not Determined

The isolated RNA can also be analyzed on agarose gels that have been stained with ethidium bromide. For this purpose, for example, 1.2% formaldehyde agarose gels were prepared. The result is shown in Figure 6. In Figure 6, Lane 1 is the total RNA that was isolated on a hydrophobic nylon membrane (Magna SH, Sample no. 1) with a pore diameter of 1.2 µm. Lane 2 is total RNA that was isolated by means of a hydrophobic nylon membrane (Hydrolon, Sample no. 2) with a pore diameter of 1.2 µm. Lane 3 represents the chromatogram of a total RNA that was isolated by means of a silica membrane (Sample no. 4). In each case, 50 µl of the total RNA eluate was analyzed. Fig. 6 provides convincing evidence that when a silica membrane was used, no measurable proportion of the total RNA can be isolated.

Example 2: Isolation of free RNA by binding the RNA to hydrophobic membranes by means of various salt-alcohol mixtures

In this example, the lysate and washing solutions are conducted through the hydrophobic membrane by applying a vacuum.

Hydrophobic nylon membranes (e.g., 1.2 µm Hydrolon from Pall) were introduced into plastic columns connected to a vacuum chamber, in a manner similar to that of Example 1. To 100 µl aliquots of an aqueous solution containing total RNA were added 350µl of a commercially available lysis buffer containing guanidium isothiocyanate (e.g., RLT buffer from QIAGEN), 350 µl of 1.2 M sodium acetate solution, or 350 µl of 4 M lithium chloride solution, respectively, and the resulting solutions were mixed by pipetting.

Next, 250 µl of ethanol were added to each mixture and mixed, likewise by pipetting. After that, the solutions containing RNA were transferred into the plastic columns and suctioned through the membrane by evacuating the vacuum chamber. Under the conditions described, the RNA remains bound to the membranes. The membranes were then washed, as described in Example 1. Finally, the RNA, also as described in Example 1, was removed from the membrane by pipetting from above.

The quantity of isolated total RNA was determined by spectrophotometric measurement of the light absorption at 260 nm. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. The results are set forth in Table 2 below.

Table 2: Isolation of RNA from aqueous solution by binding the RNA to hydrophobic membranes using various salt-alcohol mixtures.

Sample No.	Salt/Alcohol mixture	Yield of Total RNA (µg)	Absorbance E_{260}/E_{280}
1	RLT-Buffer QIAGEN/35% Ethanol	9.5	1.92
2	0.6 M Sodium Acetate/35% Ethanol	8.5	1.98
3	1 M Sodium Chloride/35% Ethanol	7.9	1.90
4	2 M Lithium Chloride/35% Ethanol	4.0	2.01

Example 3: Isolation of total RNA from HeLa cells

Following the procedures of Example 1, plastic columns were assembled with different hydrophobic membranes. Each column thus prepared was placed in a collection tube, and the following isolation steps were performed by way of centrifugation.

For the isolation, $5x10^5$ HeLa cells were harvested by centrifugation and the supernatant removed. The cells were lysed by the addition of 150 µl of a commercially available guanidinium isothiocyanate buffer, such as, e.g., RLT-buffer from QIAGEN, using well known procedures. In this connection, lysis is encouraged by multiple pipetting or by vortexing for 5 seconds. Subsequently, 150 µl of 70% ethanol was added and mixed by multiple pipetting or by vortexing for 5 seconds.

The lysate was subsequently transferred into a plastic column and passed through the membrane by centrifugation at 10000 x g for 1 minute. Subsequently, washing was performed with a commercially available washing buffer containing guanidinium isothiocyanate, e.g., with the RW1-buffer of QIAGEN, followed by a second washing step using a buffer containing TRIS and alcohol, e.g., RPE-buffer from QIAGEN. The washing buffers were passed through the membrane by centrifugation. The last washing step takes place at 20000 x g for 2 minutes to dry the membrane.

For elution, 70 µl of RNase-free water were pipetted onto the membrane to release the purified RNA from the membrane. After a 1–2 minute incubation at a temperature between 10° – 30°C, the eluate was taken from above by pipetting from the membrane. The elution step was repeated once to achieve complete elution.

The quantity of isolated total RNA was determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm. RNA quality is determined by spectrophotometric determination of the light absorption ratio compared at 260 nm and at 280 nm. The isolation results with different hydrophobic membranes are listed in Table 3 below. The data represent the average of 3–5 parallel tests per membrane. Using a silica membrane, no measurable quantity of total RNA could be isolated, where the eluate was recovered by removing it from above from the membrane.

Table 3: Yield of total RNA isolated by binding to hydrophobic membranes.

Manufacturer	Membrane	Material	RNA(μg)	E_{260}/E_{280}
Pall	Hydrolon, 1.2 μm	Hydrophobic Nylon	6.53	1.7
Pall	Hydrolon, 3 μm	Hydrophobic Nylon	9.79	1.72
Pall	Fluoro Trans G	Hydrophobic Polyvinylidene Fluoride	6.16	1.72
Pall	Fluoro Trans W	Hydrophobic Polyvinylidene Fluoride	5.4	1.9
Pall	Bio Trace	Hydrophobic Polyvinylidene Fluoride	4.3	1.97
Pall	Supor-450 PR	Hydrophobic Polyethersulfone	3.96	1.76
Pall	V-800 R	Hydrophobic Acryliccopolymer	6.26	1.72
Pall	Versapor – 1200R	Hydrophobic Acryliccopolymer	6.23	1.68
Pall	Versapor – 3000R	Hydrophobic Acryliccopolymer	3.54	1.74
Gore-Tex	OH 9335	Hydrophobic Poly-Tetrafluoroethylene	1.59	1.72
Gore-Tex	OH 9336	Hydrophobic Poly-Tetrafluoroethylene	2.15	1.65
Gore-Tex	OH 9337	Hydrophobic Poly-Tetrafluoroethylene	3.6	1.59
Gore-Tex	QH 9316	Hydrophobic Poly-Tetrafluoroethylene	3.61	1.69
Gore-Tex	QH 9317	Hydrophobic Poly-Tetrafluoroethylene	2.87	1.70
Millipore	Mitex Membrane	Hydrophobic Poly-Tetrafluoroethylene	1.98	1.62
Millipore	Durapore	Hydrophobic Polyviylidene Fluoride	7.45	1.72
MSI	Magna-SH, 1.2 μm	Hydrophobic Nylon	4.92	1.69

MSI	Magna-SH, 5 µm	Hydrophobic Nylon	10.2	1.71
MSI	Magna-SH, 10 µm	Hydrophobic Nylon	7.36	1.76
MSI	Magna-SH, 20 µm	Hydrophobic Nylon	7.04	1.65
Sartorius	Type 118	Hydrophobic Poly-Tetrafluoroethylene	7.6	1.61
Mupor	PM12A	Hydrophobic Poly-Tetrafluoroethylene	6.7	1.77
Mupor	PM3VL	Hydrophobic Poly-Tetrafluoroethylene	6.6	1.77

Example 3b: Isolation of total RNA from HeLa-cells by binding to hydrophilic membranes

Using the procedures of Example 1, plastic columns were assembled using different hydrophilic membranes. Each column thus prepared was placed in a collection tube, and the following isolation steps were performed by centrifugation.

For the isolation, $5x10^5$ HeLa cells were used. The isolation steps and elution of the nucleic acids were carried out as described above in Example 3 for hydrophobic membrane columns.

The quantity of isolated total RNA was determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm. RNA quality was determined by the spectrophotometric determination of the ratio of the light absorption compared at 260 nm and at 280 nm. The isolation results with various hydrophilic membranes are listed in Table 3b below. The data represent the average of 2–5 parallel tests per membrane. Using a silica membrane, no measurable quantity of total RNA could be isolated, where the eluate was recovered by removing it from above from the membrane.

Table 3b: Yield of total RNA isolated by binding to hydrophilic membranes.

Manufacturer	Membrane	Material	RNA (μg)	E_{260}/E_{280}
Pall	Loprodyne	Hydrophilic Nylon	3.1	1.8
Pall	Loprodyne	Hydrophilic Nylon	3.1	1.78
Pall	Biodyne A	Hydrophilic Nylon	3.1	1.8
Pall	Biodyne A	Hydrophilic Nylon	3.6	1.83
Pall	Biodyne B	Hydrophilic Nylon	2.6	1.84
Pall	Biodyne B	Hydrophilic Nylon	4.2	1.84
Pall	Biodyne C	Hydrophilic Nylon	6.1	1.88
Pall	Biodyne C	Hydrophilic Nylon	5.2	1.91
Pall	Biodyne plus	Hydrophilic Nylon	3.3	1.87
Pall	I.C.E.–450	Hydrophilic Polyethersulfone	6.36	1.8
Pall	I.C.E.-450sup	Hydrophilic Polyethersulfone	3.07	1.71
Pall	Supor – 800	Hydrophilic Polyethersulfone	4.12	1.7
Pall	Supor – 450	Hydrophilic Polyethersulfone	4.69	1.69
Pall	Supor – 100	Hydrophilic Polyethersulfone	3.25	1.71
Pall	Hemasep V	Hydrophilic Polyester	4.16	1.74
Pall	Hemasep L	Hydrophilic Polyester	6.67	1.65
Pall	Leukosorb	Hydrophilic Polyester	1.5	1.84
Pall	Premium Release	Hydrophilic Polyester Membrane	1.66	1.63
Pall	Polypro –450	Hydrophilic Polypropylene	5.09	1.78
Gore-Tex	OH 9339	Hydrophilic Poly-Tetrafluoroethylene	1.08	1.65
Gore-Tex	OH 9338	Hydrophilic Poly-Tetrafluoroethylene	3.97	1.67
Gore-Tex	QH 9318	Hydrophilic Poly-Tetrafluoroethylene	3.61	1.69
Millipore	Durapore	Polyvinylidene Fluoride made	5.6	1.69

		Hydrophilic		
Millipore	Durapore	Polvinylidene Fluoride made Hydrophilic	3.12	1.68
Millipore	LCR	Poly-Tetrafluoroethylene made Hydrophilic	3.14	1.66
Sartorius	Type 250	Hydrophilic Polyamide	4.3	1.66
Sartorius	Type 113	Hydrophilic Cellulose Nitrate	1.8	1.86
Sartorius	Type 113	Hydrophilic Cellulose Nitrate	1.9	1.74
Infiltec	Polycone, 0.01	Hydrophilic Polycarbonate	0.17	1.64
Infiltec	Polycone, 0.1	Hydrophilic Polycarbonate	0.73	1.68
Infiltec	Polycone, 1	Hydrophilic Polycarbonate	3.33	1.86

Example 4: Isolation of free RNA from an aqueous solution

Using the procedures according to Example 1, plastic columns were assembled with different hydrophobic membranes. 100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a commercially available lysis buffer containing guanidinium-isothiocyanate, e.g., RLT-buffer from QIAGEN. Subsequently, 250 µl of ethanol were added and mixed by pipetting. This mixture was then introduced to the column and passed through by centrifugation (10000 x g; 1 minute) through the membrane. The membranes were subsequently washed twice with a washing buffer, e.g., RPE from QIAGEN. The buffer was passed through the membranes by centrifugation. The last washing step was carried out at 20000 x g to dry the membranes.

Next, the RNA, as described in Example 1, was eluted with RNase-free water and removed from the membrane from above by pipetting. The quantity of isolated total RNA was determined by spectrophotometric measurement of light absorption at a wavelength of 260 nm. RNA quality was determined by the spectrophotometric determination of the ratio of the light absorption at 260 nm to 280 nm. The isolation results with various hydrophobic membranes are listed in Table 4 below. The data represent the average of 3–5 parallel tests per membrane.

Using a silica membrane, no measurable quantity of total RNA could be isolated, where the eluate was recovered by removing it from above from the membrane.

Table 4: Isolation of free RNA from an aqueous solution by binding to hydrophobic membranes.

Manufacturer	Membrane	Material	RNA (µg)	E_{260}/E_{280}
Pall	Hydrolon, 1.2 µm	Hydrophobic Nylon	5.15	1.75
Pall	Hydrolon, 3 µm	Hydrophobic Nylon	0.22	1.79
Pall	Fluoro Trans G	Hydrophobic Polyvinylidene Fluoride	5.83	1.79
Pall	Fluoro Trans W	Hydrophobic Polyvinylidene Fluoride	5.4	1.84
Pall	Bio Trace	Hydrophobic Polyvinylidene Fluoride	4.0	1.79
Pall	Emflon	Hydrophobic Poly-Tetrafluor-Ethylene	0.2	1.7
Pall	Supor-450 PR	Hydrophobic Polyethersulfone	5.97	1.71
Pall	Supor-200 PR	Hydrophobic Polyethersulfone	2.83	1.66
Pall	V-800 R	Hydrophobic Acrylatecopolymer	2.74	1.77
Gore-Tex	OH 9335	Hydrophobic Poly-Tetrafluor-Ethylene	4.35	1.63
Gore-Tex	OH 9336	Hydrophobic Poly-Tetrafluor-Ethylene	7.43	1.71
Gore-Tex	OH 9337	Hydrophobic Poly-Tetrafluor-Ethylene	5.96	1.62
Gore-Tex	QH 9316	Hydrophobic Poly-Tetrafluor-Ethylene	5.92	1.67
Gore-Tex	QH 9317	Hydrophobic Poly-Tetrafluor-Ethylene	8.7	1.66
Millipore	Fluoropore	Hydrophobic Poly-Tetrafluor-Ethylene	8.46	1.70
Millipore	Durapore, 0.65 µm	Hydrophobic Polyvinylidene Fluoride	4.23	1.8
MSI	Magna-SH, 1.2 µm	Hydrophobic Nylon	1.82	1.76
MSI	Magna-SH, 5 µm	Hydrophobic Nylon	0.6	1.78
Sartorius	Type 118	Hydrophobic Poly-Tetrafluor-Ethylene	0.9	1.82
Sartorius	Type 118	Hydrophobic Poly-Tetrafluor-Ethylene	5.4	1.74
Mupor	PM12A	Hydrophobic Poly-Tetrafluor-Ethylene	1.1	1.98

Example 4b: Isolation of free RNA from an aqueous solution by binding to hydrophilic membranes

Following the procedures of Example 1, plastic columns were assembled using different hydrophilic membranes.

100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a commercially available lysis buffer containing guanidinium-isothiocyanate, e.g., RLT-buffer from QIAGEN. Subsequently 250 µl of ethanol were added and mixed by pipetting back and forth. This mixture was then introduced to the column, passed through the membrane, washed and dried according to the procedure used in Example 4, above.

Finally, the RNA, as described in Example 1, was eluted with RNase-free water and removed from the membrane using a pipette.

The quantity of isolated total RNA was determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm. RNA quality was determined by the spectrophotometric determination of the ratio of the light absorption compared at 260 nm and at 280 nm. The isolation results with various hydrophilic membranes are listed in Table 4b below. The data represent the average from 2–5 parallel tests per membrane. Using a silica membrane, no measurable quantity of total RNA could be isolated, where the eluate was recovered by removing it from above from the membrane.

Table 4b: Isolation of free RNA from an aqueous solution by binding to hydrophilic membranes.

Manufacturer	Membrane	Material	RNA (µg)	E_{260}/E_{280}
Pall	Loprodyne	Hydrophilic Nylon	2	1.8
Pall	Loprodyne	Hydrophilic Nylon	1.4	1.87
Pall	Biodyne A	Hydrophilic Nylon	4.5	1.93
Pall	Biodyne A	Hydrophilic Nylon	3.1	1.9
Pall	Biodyne B	Hydrophilic Nylon	1.7	1.94
Pall	Biodyne B	Hydrophilic Nylon	1.2	1.94
Pall	Biodyne C	Hydrophilic Nylon	3.7	1.93
Pall	Biodyne C	Hydrophilic Nylon	3.1	1.93
Pall	Biodyne plus	Hydrophilic Nylon	1.1	1.87

Pall	I.C.E.–450	Hydrophilic Polyethersulfone	1.92	1.82
Pall	I.C.E.-450sup	Hydrophilic Polyethersulfone	0.87	1.67
Pall	Supor – 800	Hydrophilic Polyethersulfone	3.93	1.74
Pall	Supor – 450	Hydrophilic Polyethersulfone	1.78	1.74
Pall	Supor – 100	Hydrophilic Polyethersulfone	1.04	1.68
Pall	Hemasep V	Hydrophilic Polyester	4	1.79
Pall	Hemasep L	Hydrophilic Polyester	0.47	2.1
Pall	Polypro – 450	Hydrophilic Polypropylene	5.09	1.78
Gore-Tex	OH 9339	Hydrophilic Poly-Tetrafluor-Ethylene	0.43	1.48
Gore-Tex	OH 9338	Hydrophilic Poly-Tetrafluor-Ethylene	3.63	1.64
Gore-Tex	QH 9318	Hydrophilic Poly-Tetrafluor-Ethylene	5.92	1.67
Millipore	Durapore	Polyvinylidene Fluoride made Hydrophilic	1.18	1.79
Millipore	LCR	Poly-Tetrafluor-Ethylene made Hydrophilic	2.84	1.72
Sartorius	Type 250	Hydrophilic Polyamide	2.7	1.7
Sartorius	Type 111	Hydrophilic Cellulose Acetate	1.6	1.85
Sartorius	Type 111	Hydrophilic Cellulose Acetate	2.2	2.1
Sartorius	Type 111	Hydrophilic Cellulose Acetate	0.3	2.01
Sartorius	Type 113	Hydrophilic Cellulose Nitrate	4	1.88
Sartorius	Type 113	Hydrophilic Cellulose Nitrate	3.8	1.87

Example 5: Isolation of total RNA from HeLa-cells depending on the pore size of the membranes

Following the procedures of Example 1, plastic columns were assembled with different hydrophobic membranes with different pore sizes.

As in Example 3, a cell lysate was made from $5x10^5$ HeLa cells and transferred to the columns. Subsequently the membranes were washed with the commercially available buffers RW1 and RPE from QIAGEN. The last centrifugation step was carried out at 20000 x g for 2 minutes to dry the membrane. The elution was carried out as described in Example 1.

The results are listed in Table 5 below. 3–5 parallel tests per membrane were performed and the average value calculated for each.

Table 5: Yield of isolated total RNA using hydrophobic membranes with different pore sizes.

Manufacturer	Membrane	Material	Pore Size (µm)	RNA (µg)	E_{260}/E_{280}
Infiltec	Polycon 0.01	Hydrophilic Polycarbonate	0.01	0.17	1.64
Pall	Fluoro Trans G	Hydrophobic Polyvinylidene Fluoride	0.2	6.16	1.72
Pall	Supor-450 PR	Hydrophobic Polyethersulfone	0.45	3.96	1.76
Millipore	Durapore	Hydrophobic Polyvinylidene Fluoride	0.65	7.45	1.72
MSI	Magna-SH	Hydrophobic Nylon	1.2	4.92	1.69
MSI	Magna-SH	Hydrophobic Nylon	5	10.2	1.71
MSI	Magna-SH	Hydrophobic Nylon	10	7.36	1.76
MSI	Magna-SH	Hydrophobic Nylon	20	7.04	1.65

Example 6: Stability and quality of isolated total RNA from HeLa cells

According to procedures of Example 1, plastic columns were assembled with a commercially available membrane (Pall, Hydrolon with a 3 µm pore size).

According to the procedures of Example 3, a cell lysate was made from $5x10^5$ HeLa cells and transferred to the columns. Subsequently, the membranes were washed with the commercially available buffers RW1 and RPE from QIAGEN. The last centrifugation step was carried out at 20000 x g for 2 minutes to dry the membrane. The elution was carried out as described in Example 1.

The isolated total RNA was left to incubate for 16 hours at 37°C and subsequently placed on a denaturating agarose gel and analyzed. It was demonstrated that the RNA did not suffer degradation. The RNA isolated with the method described above shows no contaminants with enzymes that degrade nucleic acids and therefore is of high quality.

Example 7: Isolation of free RNA from an aqueous solution by binding to a hydrophilic membrane in a 96-well plate

A 96-well plate with a hydrophilic Polyvinylidene Fluoride membrane (Durapore, 0.65 µm by Millipore) was used. 5.3 ml of an aqueous solution containing total RNA were mixed with 18.4 ml of a commercially available lysis buffer containing guanidinium isothiocyanate, e.g., RLT buffer from QIAGEN. Subsequently 13.1 ml ethanol were added and mixed by pipetting back and forth. For each well, 350 µl of this mixture were introduced and passed through the membrane by applying a vacuum. The membranes were subsequently washed twice with a buffer, e.g., RPE from QIAGEN. The buffer was passed through the membrane each time by applying a vacuum. After the last washing step, the plate was dabbed once with a paper towel and subsequently dried for 5 minutes by applying a vacuum.

The RNA was eluted as described in Example 1, with RNase-free water and removed from the membrane by way of a pipette. The quantity of isolated total RNA was determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm and the average value as well as the standard deviation for the entire plate was calculated. The average value is 8.4 µg with a standard deviation of 0.7 µg.

Example 8: Isolation of total RNA by way of capillary forces

A 96-well plate with a hydrophilic Polyvinylidene Fluoride membrane (Durapore, 0.65 µm by Millipore) was used. 33 µl of an aqueous solution containing total RNA were mixed with 110 µl of a commercially available lysis buffer containing guanidinium isothiocyanate, e.g., RLT

buffer from QIAGEN. Subsequently 78 µl ethanol were added and mixed by pipetting. 45 µl of this mixture were introduced into each well. An absorbent household sponge was moistened with water, and the 96-well plate was placed with the membrane's bottom side on the sponge. The RNA mixture was passed through the membrane by way of capillary forces. The membranes were subsequently washed twice with a buffer, e.g., RPE from QIAGEN. The wash buffer was also passed through the membrane by placing the plate on the sponge. After the last washing step, the plate was air-dried for 5 minutes.

The RNA, as described in Example 1, was eluted with RNase-free water and removed from the membrane by way of a pipette.

The quantity of isolated total RNA is subsequently determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm, and the average value as well as the standard deviation is calculated. The average value is 5.9 µg with a standard deviation of 0.7 µg.

Example 9: Isolation of genomic DNA from an aqueous solution by way of a buffer containing guanidinium hydrochloride

According to Example 1, plastic columns were assembled with hydrophobic membranes (e.g., Magna-SH, 5 µm by the MSI Company). Purification is carried out with commercially available buffers from QIAGEN.

200 µl of an aqueous solution of genomic DNA from liver tissue were introduced in PBS buffers. 200 µl of a buffer containing guanidinium hydrochloride, e.g. QIAGEN's AL, were added to and mixed with this solution. Subsequently 210 µl of ethanol were added and mixed through vortexing. The mixture was introduced to the column according to Example 3 and passed through the membrane by way of centrifugation. The membrane was then washed and dried with an alcohol containing buffer, e.g., QIAGEN's AW. The elution was performed as described in Example 1. Three parallel tests were carried out and the average value calculated. The amount of isolated DNA is subsequently determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm and is approx. 30% of the starting amount. The absorption ratio at 260 nm to 280 nm is 1.82.

Example 10: Isolation of genomic DNA from an aqueous solution by binding to hydrophobic membranes by way of a buffer containing guanidinium isothiocyanate

According to Example 1, plastic columns were assembled with different membranes. 100 µl of an aqueous solution containing total DNA were mixed with 350 µl of a lysis buffer containing guanidinium isothiocyanate (4 M GITC, 0.1 M $MgSO_4$, 25 mM Na-Citrate, pH 4). Subsequently 250 µl ethanol were added and mixed by pipetting. This mixture was then transferred to the column and passed through the membrane by way of centrifugation (10000 x g; 1 minute). The membranes were subsequently washed twice with a buffer, e.g., RPE by QIAGEN. The buffer was passed through the membranes by way of centrifugation. The last washing step was carried out at 20000 x g to dry the membranes.

The elution was performed as described in Example 1. Three parallel tests were carried out per membrane and the average value is calculated each time. The results are listed in Table 6.

Table 6: DNA-yield from an aqueous solution by binding to hydrophobic membranes

Manufacturer	Membrane	Material	DNA (µg)
Pall	Hydrolon, 1.2 µm	Hydrophobic Nylon	1.3
Pall	Supor-450 PR	Hydrophobic Polyethersulfon	2.2
Millipore	Fluoropore	Hydrophobic Poly-Tetrafluor-Ethylene	1.1
Millipore	Durapore	Hydrophobic Polyvinylidene Fluoride	1.2

Example 11: Isolation of genomic DNA from tissue

According to Example 1, plastic columns were assembled with hydrophobic membranes (e.g., Magna-SH, 5 µm by MSI). Purification was carried out with the commercially available buffers from QIAGEN.

180 µl of ATL-buffer were added to 10 mg of kidney tissue (mouse) and ground in a mechanical homogenizer. Subsequently proteinase K (approx. 0.4 mg dissolved in 20 µl of water) were added and incubated for 10 minutes at 55°C. After adding 200 µl of a buffer containing guanidinium hydrochloride, e.g., AL by QIAGEN, and after a 10-minute incubation at

70°C, 200 µl of ethanol were added and mixed with this solution. This mixture was transferred on to the column and passed through the membrane by centrifugation. The membrane was then washed with alcohol containing buffers, e.g., AW1 and AW2 from QIAGEN, and subsequently dried by way of centrifugation. The elution was carried out as described in Example 1. Three parallel tests were carried out and the average value calculated.

The amount of isolated DNA, determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm, was on average 9.77 µg. The absorption ratio at 260 nm to 280 nm was 1.74.

Example 12: Isolation of genomic DNA from blood

According to the procedures of Example 1, plastic columns were assembled with hydrophobic membranes (e.g., Magna-SH, 5 µm by MSI). Purification was carried out with the commercially available buffers from QIAGEN.

200 µl of AL buffer and 20 µl of QIAGEN protease were added to 200 µl of blood, thoroughly mixed, and left to incubate for 10 minutes at 56°C. After adding 200 µl of ethanol, the solution was mixed, transferred onto the column, and passed through the membrane by way of centrifugation. The membrane was then washed with alcohol containing buffers, e.g., AW1 and AW2 from QIAGEN, and subsequently dried by way of centrifugation. The elution was carried out as described in Example 1.

The amount of isolated DNA, determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm, was 1.03 µg. The absorption ratio at 260 nm 280 nm is 1.7.

Example 13: Isolation of total RNA from an RNA-DNA-mixture

Following the procedures of Example 1, plastic columns were assembled with hydrophobic membranes (e.g., Hydrolon 1.2 µm by the Pall Company). 275 µl of an aqueous solution containing total RNA and genomic DNA were mixed with 175 µl of a commercially available lysis buffer containing guanidinium isothiocyanate, e.g., the RLT buffer from QIAGEN. 250 µl of ethanol were added and mixed by pipetting. The mixture was transferred to the column and passed through the membrane, washed and dried according to Example 4. The

flow-through from the first centrifugation step was placed on a commercially available mini-spin column (e.g., QIAamp Mini-Spin Column from QIAGEN) and passed through the membrane via centrifugation. The remaining washing steps were performed as described in Example 4.

After this, the nucleic acids were eluted with 140 µl of RNase-free water by way of centrifugation (10000 x g, 1 minute) and analyzed in non-denaturing agarose gel (see Figure 7). The major part of the total RNA can be separated from the genomic DNA with the use of the method described above.

Figure 7 shows an ethidium-bromide stained gel of an electrophoretic separation of two different eluates.

Lane 1: Isolation of total RNA by way of a hydrophobic nylon membrane.

Lane 2: Isolation of genomic DNA from the flow-through by way of a QIAamp mini-spin column of the QIAGEN company.

Example 14: Isolation of plasmid DNA from an aqueous solution by binding to hydrophobic and hydrophilic membranes

Following the procedures of Example 1, plastic columns were assembled utilizing different membranes.

100 µl of an aqueous solution (pCMVβ from Clontech) containing plasmid were mixed with 350 µl of lysis buffer containing guanidinium isothiocyanate (4 M GITC, 0.1 M $MgSO_4$, 25 mM sodium-acetate, pH 4). Subsequently, 250 µl of isopropanol were added and mixed by pipetting. This mixture was then transferred onto one of the columns and passed through the membrane, washed and dried according to the procedures described in Example 4. Finally the plasmid DNA, as described previously in Example 1, was eluted with RNase-free water and removed from the membrane by pipetting.

The amount of isolated plasmid DNA was determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm. The isolation results using various membranes are listed in Table 7 below. Three parallel tests per membrane were carried out and each time the average value is calculated.

Table 7: Plasmid DNA-yield from an aqueous solution by binding to membranes

Manufacturer	Membrane	Material	Plasmid DNA (µg)
Pall	Hydrolon, 1.2 µm	Hydrophobic Nylon	1.9
Pall	Fluoro Trans G	Hydrophobic Polyvinylidene Fluoride	2.2
Pall	I.C.E.-450	Hydrophilic Polyethersulfone	0.8
Pall	I.C.E.-450sup	Hydrophilic Polyethersulfone	1.5
Pall	Supor-450 PR	Hydrophobic Polyethersulfone	4.7
Pall	Supor-200 PR	Hydrophobic Polyethersulfone	4
Pall	Supor-800	Hydrophilic Polyethersulfone	0.5
Pall	Supor-450	Hydrophilic Polyethersulfone	0.9
Pall	Supor-100	Hydrophilic Polyethersulfone	1
Pall	V-800 R	Hydrophobic Acrylic Copolymer	1.5
Pall	Versapore-1200R	Hydrophobic Acrylic Copolymer	0.2
Pall	Polypro-450	Hydrophilic Polypropylene	1.4
Gore-Tex	QH 9318	Hydrophilic Poly-Tetrafluoro-Ethylene	4.9
Gore-Tex	OH 9335	Hydrophobic Poly-Tetrafluoro-Ethylene	4.3
Millipore	Durapore, 0.65 µm	Polyvinylidene Fluoride made Hydrophobic	1.8
Millipore	Durapore, 0.65 µm	Hydrophobic Polyvinylidene Fluoride	1.7
MSI	Magna-SH, 1.2 µm	Hydrophobic Nylon	1.1

Example 15: Immobilization of total RNA from an aqueous solution with the use of different chaotropic agents

Following the procedures of Example 1, plastic columns were assembled utilizing different hydrophobic membranes.

100 µl of an aqueous solution containing total RNA were mixed with 350 µl of different lysis buffers, which contain guanidinium isothiocyanate (GITC) or guanidinium hydrochloride

(GuHCl) in different concentrations. 250 µl ethanol were added and mixed by pipetting. This mixture was then placed on one of the columns and passed through the membrane by way of centrifugation (10000 x g; 1 minute). The membranes were subsequently washed twice with an alcohol containing buffer, e.g., RPE from QIAGEN. The buffer was passed through the membrane by centrifugation. The last washing step was performed at 20000 x g to dry the membrane. The elution was carried out as described in Example 1. Two tests were carried out to determine the average value. The results are listed in Table 8.

Table 8: RNA-yield from an aqueous solution by way of chaotropic agents

Membrane	Chaotropic Agents, Concentration in Binding Solution	Yield of Total RNA (µg)
Hydrolon, 1.2 µm	GITC, 500 mM	2.3
Hydrolon, 1.2 µm	GITC, 1 M	0.8
Hydrolon, 1.2 µm	GITC, 3 M	0.9
Fluoro Trans G	GITC, 500 mM	0.4
Fluoro Trans G	GITC, 1 M	1.25
Fluoro Trans G	GITC, 3 M	0.6
Hydrolon, 1.2 µm	GuHCl, 500 mM	2.6
Hydrolon, 1.2 µm	GuHCl, 1 M	6.7
Hydrolon, 1.2 µm	GuHCl, 3 M	2.9
Fluoro Trans G	GuHCl, 500 mM	0.4
Fluoro Trans G	GuHCl, 1 M	1.25
Fluoro Trans G	GuHCl, 3 M	0.6

Example 16: Immobilization of total RNA from an aqueous solution using alcohols

Following the procedures of Example 1, plastic columns were assembled utilizing different hydrophobic membranes. 100 µl of an aqueous solution containing total RNA are mixed with 350 µl of a lysis buffer containing guanidinium isothiocyanate (concentration 4 M). Different amounts of ethanol and isopropanol were added and filled with RNase-free water up to

700 µl and mixed. This mixture was then introduced to a column and passed through the membrane and washed according to the procedures of Example 4. The elution took place as in Example 1. Two tests were carried out to determine the average yield. The results are listed in Table 9.

Table 9: RNA-yield from an aqueous solution with different alcohols in a binding solution

Membrane	Alcohol, Concentration in Binding Solution	Yield of Total RNA (µg)
Hydrolon, 1.2 µm	Ethanol, 5%	0.7
Hydrolon, 1.2 µm	Ethanol, 30%	2.85
Hydrolon, 1.2 µm	Ethanol, 50%	4.5
Durapore, 0.65 µm	Ethanol, 5%	0.4
Durapore, 0.65 µm	Ethanol, 30%	1.25
Durapore, 0.65 µm	Ethanol, 50%	0.6
Hydrolon, 1.2 µm	Isopropanol, 5%	0.35
Hydrolon, 1.2 µm	Isopropanol, 30%	4.35
Hydrolon, 1.2 µm	Isopropanol, 50%	3.2
Durapore, 0.65 µm	Isopropanol, 10%	1.35
Durapore, 0.65 µm	Isopropanol, 30%	4.1
Durapore, 0.65 µm	Isopropanol, 50%	3.5

Example 17: Immobilization of total RNA from an aqueous solution with various pH-values

Using the procedures described in Example 1, plastic columns were assembled utilizing various hydrophobic membranes. 100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a lysis buffer containing guanidinium isothiocyanate (concentration 4 M). The buffer contained 25 mM of sodium citrate and was adjusted to different pH-values with HCl or NaOH. Subsequently, 250 µl of ethanol were added and mixed. This mixture was then introduced to the column and passed through the membrane and washed according to the

procedures of Example 4. The elution took place as in Example 1. Two tests are carried out to determine an average value. The results are listed in Table 10.

Table 10: RNA-yield from an aqueous solution with various pH-values in a binding solution

Membrane	pH of Binding Solution	Yield of Total RNA (µg)
Hydrolon, 1.2 µm	pH 3	0.15
Hydrolon, 1.2 µm	pH 9	1.6
Hydrolon, 1.2 µm	pH 11	0.05
Fluoro Trans G	pH 1	0.45
Fluoro Trans G	pH 9	2.85
Fluoro Trans G	pH 11	0.25

Example 18: Immobilization of total RNA from an aqueous solution with various salts

According to Example 1, plastic columns are assembled with hydrophobic membranes. 100 µl of a total RNA containing aqueous solution were mixed with 350 µl of a salt containing lysis buffer (NaCl, KCL, $MgSO_4$). 250 µl of H_2O or ethanol were then added and mixed. This mixture was then transferred to a column and passed through the membrane, washed and eluted according to the procedures of Example 4. Two tests were carried out to determine the average value. The results are listed in Table 11.

Table 11: RNA-yield from an aqueous solution with various salts in the binding solution

Membrane	Salt Concentration in Binding Solution	Yield of Total RNA (µg)
Hydrolon, 1.2 µm	NaCl, 100 mM; without ethanol	0.1
Hydrolon, 1.2 µm	NaCl, 1 M; without ethanol	0.15
Hydrolon, 1.2 µm	NaCl, 5 M; without ethanol	0.3
Hydrolon, 1.2 µm	KCl, 10 mM; without ethanol	0.2
Hydrolon, 1.2 µm	KCl, 1 M; without ethanol	0.1
Hydrolon, 1.2 µm	KCl, 3 M; without ethanol	0.25

Hydrolon, 1.2 µm	$MgSO_4$, 100 mM; without ethanol	0.05
Hydrolon, 1.2 µm	$MgSO_4$, 750 mM; without ethanol	0.15
Hydrolon, 1.2 µm	$MgSO_4$, 2 M; without ethanol	0.48
Hydrolon, 1.2 µm	NaCl, 500 mM; with ethanol	2.1
Hydrolon, 1.2 µm	NaCl, 1 M; with ethanol	1.55
Hydrolon, 1.2 µm	NaCl, 2.5 M; with ethanol	1.35
Hydrolon, 1.2 µm	KCl, 500 mM; with ethanol	1.6
Hydrolon, 1.2 µm	KCl, 1 M; with ethanol	2.1
Hydrolon, 1.2 µm	KCl, 1.5 M; with ethanol	3.5
Hydrolon, 1.2 µm	$MgSO_4$, 10 mM; with ethanol	1.9
Hydrolon, 1.2 µm	$MgSO_4$, 100 mM; with ethanol	4.6
Hydrolon, 1.2 µm	$MgSO_4$, 500 M; with ethanol	2

Example 19: Immobilization of total RNA from an aqueous solution using various buffer conditions

Following the procedures of Example 1, plastic columns were assembled using different hydrophobic membranes.

100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a lysis buffer containing guanidinium isothiocyanate (concentration 2.5 M). The lysis buffer was mixed with various concentrations of sodium citrate, pH 7, or sodium oxalate, pH 7.2. Subsequently 250 µl of ethanol were added and mixed. This mixture was then transferred to a column and passed through the membrane and eluted according to the process described in Example 4. The results are listed in Table 12. Two tests were carried out to determine the average value.

Table 12: RNA-yield from an aqueous solution with various buffer concentrations in a binding solution

Membrane	Na-Citrate/Na-Oxalate, Conc. in Lysis Buffer	Yield of Total RNA (μg)
Hydrolon, 1.2 μm	Na-Citrate, 10 mM	2.2
Hydrolon, 1.2 μm	Na-Citrate, 100 mM	2.4
Hydrolon, 1.2 μm	Na-Citrate, 500 mM	3.55
Supor-450 PR	Na-Citrate, 10 mM	1.1
Supor-450 PR	Na-Citrate, 100 mM	1.15
Supor-450 PR	Na-Citrate, 500 mM	0.2
Hydrolon, 1.2 μm	Na-Oxalate, 1 mM	1.5
Hydrolon, 1.2 μm	Na-Oxalate, 25 mM	1.05
Hydrolon, 1.2 μm	Na-Oxalate, 50 mM	0.9
Supor-450 PR	Na-Oxalate, 1 mM	1.9
Supor-450 PR	Na-Oxalate, 25 mM	1.3
Supor-450 PR	Na-Oxalate, 50 mM	1.7

Example 20: Immobilization of total DNA from an aqueous solution using various buffers

According to the procedures of Example 1, plastic columns were assembled with hydrophobic membranes (for example Hydrolon 1.2 μm from the Pall Company).

100 μl of an aqueous solution containing total DNA were mixed with 350 μl of a lysis buffer containing guanidinium isothiocyanate (4 M GITC, 0.1 M $MgSO_4$). To this lysis buffer various buffer substances were added (concentration 25 mM) and adjusted to different pH-values. Subsequently, 250 μl of ethanol were added and mixed. The mixture was then introduced to the column and passed through the membrane, washed and eluted as in Example 4.

The results are set forth in Table 13. Triple tests are carried out and average values determined.

Table 13: DNA-yield from an aqueous solution with various buffer substances in a binding solution

Buffer Substance	pH in the Lysis Buffer	Yield of DNA (μg)
Sodium Citrate	pH 4	1.3
Sodium Citrate	pH 5	0.6
Sodium Citrate	pH 6	1.4
Sodium Citrate	pH 7	0.5
Sodium Acetate	pH 4	0.9
Sodium Acetate	pH 5	1
Sodium Acetate	pH 6	0.6
Sodium Acetate	pH 7	0.5
Potassium Acetate	pH 4	0.6
Potassium Acetate	pH 5	0.9
Potassium Acetate	pH 6	1.2
Potassium Acetate	pH 7	1.4
Ammonium Acetate	pH 4	0.7
Ammonium Acetate	pH 5	0.3
Ammonium Acetate	pH 6	5.7
Ammonium Acetate	pH 7	1.5
Glycine	pH 4	0.5
Glycine	pH 5	1.1
Glycine	pH 6	1.6
Glycine	pH 7	1.1
Malonate	pH 4	1.5
Malonate	pH 5	0.3
Malonate	pH 6	3.1
Malonate	pH 7	1.6
Succinate	pH 4	2.8
Succinate	pH 5	2.3

Succinate	pH 6	2.5
Succinate	pH 7	4.7

Example 21: Immobilization of total RNA from an aqueous solution using phenol

According to the procedures of Example 1, plastic columns were assembled with hydrophobic membranes (e.g., Hydrolon, 1.2 µm from the Pall Company).

An aqueous solution containing RNA was mixed with 700 µl of phenol and passed through the membranes using centrifugation. The membranes were washed and the RNA eluted as in Example 4. Two tests were carried out and an average value determined.

The amount of isolated RNA was subsequently determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm and is on average 10.95 µg. The absorption ratio at 260 nm to the one at 280 nm is 0.975.

Example 22: Washing of immobilized total RNA under different salt concentrations

Following the procedures of Example 1, plastic columns were assembled with hydrophobic membranes.

100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a lysis buffer containing guanidinium isothiocyanate (concentration 4 M). Subsequently, 250 µl of ethanol were added and mixed. This mixture was then transferred to the column and passed through the membrane and washed according to Example 4. The membranes were then washed twice with a buffer containing various concentrations of NaCl and 80% ethanol. The buffer was passed through the membrane by centrifugation. The last washing step was carried out at 20000 x g in order to dry the membranes. The elution takes place according to the procedure of Example 1. Two tests were carried out and an average value determined. The results are listed in Table 14.

Table 14: RNA-yield from an aqueous solution with NaCl in the washing buffer

Membrane	NaCl in the Washing Buffer	Yield of Total RNA (µg)
Hydrolon, 1.2 µm	NaCl, 10 mM	1.4
Hydrolon, 1.2 µm	NaCl, 50 mM	3.15
Hydrolon, 1.2 µm	NaCl, 100 mM	3
Durapore, 0.65 µm	NaCl, 10 mM	2.7
Durapore, 0.65 µm	NaCl, 50 mM	2.85
Durapore, 0.65 µm	NaCl, 100 mM	2.7

Example 23: Elution of immobilized total RNA under different salt and buffer conditions

According to the procedures of Example 1, plastic columns were assembled with hydrophobic membranes.

100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a lysis buffer containing guanidinium isothiocyanate (concentration 4 M). Subsequently, 250 µl of ethanol were added and mixed. This mixture was then introduced to the column and passed through the membrane and washed according to the procedures of Example 4.

For elution, 70 µl of a NaCl-containing solution, a Tris/HCl buffer (pH 7) or a sodium oxalate solution (pH 7.2) were pipetted onto the membrane, in order to elute the purified RNA from the membrane. After 1 to 2 minutes of incubation at a temperature of 10°C – 30°C, the eluate was pipetted from above from the membrane. The elution step was repeated once in order to achieve complete elution. Two tests were carried out and an average value determined. The results are summarized in Table 15.

Table 15: RNA-yield from an aqueous solution with NaCl, Tris/HCl or sodium oxalate in the elution buffer

Membrane	NaCl or Tris in the Elution Buffer	Yield of Total RNA (µg)
Hydrolon, 1.2 µm	NaCl, 1 mM	1.35
Hydrolon, 1.2 µm	NaCl, 50 mM	1.2
Hydrolon, 1.2 µm	NaCl, 250 mM	0.45

Durapore, 0.65 µm	NaCl, 1 mM	0.9
Durapore, 0.65 µm	NaCl, 50 mM	0.35
Durapore, 0.65 µm	NaCl, 500 mM	0.15
Hydrolon, 1.2 µm	Tris/HCl, 1 mM	0.35
Hydrolon, 1.2 µm	Tris/HCl, 10 mM	0.75
Durapore, 0.65 µm	Tris/HCl, 1 mM	1.5
Durapore, 0.65 µm	Tris/HCl, 50 mM	1
Durapore, 0.65 µm	Tris/HCl, 250 mM	0.1
Hydrolon, 1.2 µm	Na-Oxalate, 1 mM	0.45
Hydrolon, 1.2 µm	Na-Oxalate, 10 mM	0.65
Hydrolon, 1.2 µm	Na-Oxalate, 50 mM	0.3
Durapore, 0.65 µm	Na-Oxalate, 1 mM	2
Durapore, 0.65 µm	Na-Oxalate, 10 mM	0.155
Durapore, 0.65 µm	Na-Oxalate, 50 mM	0.15

Example 24: Elution of the immobilized RNA at different temperatures

Following the procedure of Example 1, plastic columns were assembled using a hydrophobic membrane (e.g., Hydrolon, 3 µm from the Pall Company).

For isolation, $5x10^5$ HeLa-cells were used. The following isolation steps were carried out as described in Example 3.

For elution, 70 µl of RNase-free water of a different temperature were pipetted onto the membrane in order to elute the purified RNA from the membrane. After an incubation of 1 – 2 minutes at the corresponding elution temperature, the eluate was pipetted off the membrane from above. The elution step was repeated once in order to achieve complete elution. Triple tests were carried out and an average value determined. The results are summarized in Table 16.

Table 16: RNA-yield at different elution temperatures

Membrane	Elution Temperature	Yield of Total RNA (µg)
Hydrolon, 3 µm	Ice cold	2.2
Hydrolon, 3 µm	40°C	3.2
Hydrolon, 3 µm	50°C	3.9
Hydrolon, 3 µm	60°C	3.7
Hydrolon, 3 µm	70°C	3.7
Hydrolon, 3 µm	80°C	2.9

Example 25: Elution of immobilized RNA by way of centrifugation

Following the procedures of Example 1, plastic columns were assembled with a hydrophobic membrane (e.g., Hydrolon 1.2 µm from the Pall Company).

100 µl of an aqueous solution containing total RNA were mixed with 350 µl of a commercially available lysis buffer containing guanidinium isothiocyanate (e.g., RLT buffer from QIAGEN). 250 µl of ethanol were then added and mixed by pipetting. This mixture was then transfered onto the column and passed through the membrane using centrifugation (10000 x g; 1 minute). The membranes were subsequently washed twice with a buffer (e.g., RPE buffer from QIAGEN). Each time the buffer was passed through the membranes by way of centrifugation. The last washing step was carried out at 20000 x g in order to dry the membrane.

For elution, 70 µl of RNase-free water were pipetted onto the membrane in order to elute the RNA from the membrane. After an incubation of 1 minute at a temperature of 10°C – 30°C, the eluate was passed through the membrane by centrifugation (10000 x g, 1 minute). In order to achieve complete elution, the elution step was repeated once and the eluates joined together. Five parallel tests were carried out and the average value calculated.

The amount of isolated total RNA was subsequently determined by spectrophotometric measurement of the light absorption at a wavelength of 260 nm and was on average 6.4 µg. The absorption ratio at 260 nm to 280 nm was 1.94.

Example 26: Use of total RNA in a 'Real Time' quantitative RT-PCR using 5′ nuclease PCR assay to amplify and detect β-actin mRNA

Following the procedures of Example 3, plastic columns were assembled using a commercially available membrane (Hydrolon from Pall, with a pore size of 3 μm).

To isolate RNA, 1 x 10^5 HeLa cells were used, and the purification of total RNA was carried out as described in Example 1. The elution was carried out with 2 x 70 μl of H_2O as described in Example 1. For the complete removal of remaining amounts of DNA, the sample was treated with a DNase prior to analysis.

A "one-device 'Real Time' quantitative RT-PCR" was carried out with the use of the commercially available reaction system of Perkin-Elmer (TaqMan™ PCR Reagent Kit) by using a M-MLV reverse transcriptase. By using a specific primer and a specific TaqMan probe for β-actin (TaqMan™ β-actin Detection Kit, made by Perkin Elmer) the β-actin mRNA molecules in the total RNA sample were first converted into β-actin cDNA and subsequently the total reaction was amplified and detected immediately, without interruption, in the same reaction device. The reaction specimens were produced according to the manufacturer's instructions. Three different amounts of isolated total RNA are used (1, 2, 4 μl of eluate) and triple determination tests were carried out. As a control, three specimens without RNA were also tested.

The cDNA synthesis was carried out at 37°C for one hour, immediately followed by a PCR which comprised 40 cycles. The reactions and the analyses were carried out on an ABI PRISM™ 7700 Sequence Detector manufactured by Perkin Elmer Applied Biosystems. Every amplicon generated during a PCR-cycle produces a light emitting molecule, which is generated by splitting from the TaqMan-probe. The total light signal that is generated is directly proportional to the amplicon quantity that is being generated and hence to the original amount of transcript available in the total RNA sample. The emitted light is measured by the instrument and evaluated by a computer program. The PCR cycle, during which the light signal must first be detected over the background noise, will be designated as the "Threshold Cycle" (ct). This value is a measure for the amount of specifically amplified RNA available in the sample.

For the 1 μl RNA eluate, isolated with the process described here, an average ct-value of 17.1 was calculated; for 2 μl in total RNA the ct-value was 16.4 and for 4 μl of total RNA the ct-value was 15.3. This resulted in a linear dependency between the total RNA and the ct-value,

indicating that the total RNA was free of substances that might inhibit the amplification reaction. The control specimens containing no RNA did not produce any signals.

Example 27: Use of total RNA in an RT-PCR for amplification and detection of β-actin mRNA

According to Example 1, plastic columns were assembled with commercially available membranes (Pall, Hydrolon with a pore size of 1.2 or 3 μm; Sartorius, Sartolon with a pore size of 0.45 μm).

For isolation of RNA, two different starting materials were used: (1) total RNA from liver (mouse) in an aqueous solution; purification, elution carried out as described in Example 4; and (2) 5 x 10^5 HeLa-cells, the purification of total RNA and the elution are carried out as described in Example 3.

For each test, 20 ng of isolated total RNA were used. As a control, RNA which was purified by way of RNeasy-Kits (QIAGEN) and a sample without RNA were used.

A RT-PCR was performed with these samples under standard conditions. For amplification two different primer pairs were used for the β-actin-mRNA. A 150 bp-sized fragment serves as proof of sensitivity, a 1.7 kbp-sized fragment assesses the integrity of the RNA. From the RT-reaction, 1 μl was removed and introduced to the subsequent PCR. 25 cycles were performed for the small fragment and 27 cycles for the large fragment. The annealing temperature was 55°C. The amplified samples were subsequently placed on a non-denaturing gel and analyzed (Figure 8).

For the 20 ng quantity used of total RNA isolated in the process described above, the corresponding DNA-fragments can be demonstrated in the RT-PCR. When using total RNA from mouse liver, no transcript can be demonstrated, as the conditions used here are adjusted to human β-actin mRNA. The control specimens which contain no RNA do not produce any signals.

Figure 8 shows ethidium bromide stained agarose gels of an electrophoretic separation of RT-PCR reaction products.

Fig. 8A: Lanes 1 to 8: RT-PCR of the 150 bp fragment:

Lanes 1 & 2: RNA from mouse liver in an aqueous solution purified with the Hydrolon 1.2 μm membrane;

Lanes 3 & 4: RNA from HeLa-cells purified with the Sartolon membrane;

Lanes 5 & 6: RNA from HeLa-cells purified with the Hydrolon 3 μm membrane;

Lane 7: RNA purified using the RNeasy-Mini-Kit;

Lane 8: Control without RNA.

Fig. 8B: Lanes 1 to 8: RT-PCR of the 1.7 kbp fragment:

Lanes 1 & 2: RNA from mouse liver in an aqueous solution purified with the Hydrolon 1.2 μm membrane;

Lanes 3 & 4: RNA from HeLa-cells purified with the Sartolon membrane;

Lanes 5 & 6: RNA from HeLa-cells purified with the Hydrolon 3 μm membrane;

Lane 7: RNA purified using the RNeasy-Mini-Kit;

Lane 8: Control without RNA.

Example 28: Use of total RNA in a NASBA-reaction (Nucleic Acid Sequence Based Amplification) for the amplification and detection of β-actin mRNA

Following the procedures described in Example 1, plastic columns were assembled with commercially available membranes (Pall, Hydrolon with a pore size of 1.2 or 3 μm; Sartorius, Sartolon with a pore size of 0.45 μm).

For isolation of RNA, two different starting materials were used: (1) total RNA from liver (mouse) in an aqueous solution; purification, elution carried out as described in Example 4; and (2) 5 x 10^5 HeLa-cells, the purification of total RNA and the elution are carried out as described in Example 3.

A NASBA-reaction is performed under standard conditions (Fahy, E. et al., 1991, *PCR Methods Amplic.*, 1:25–33). For amplification, β-actin specific primers were used.

For each test 20 ng of isolated total RNA are used. As a control, RNA which was purified by way of RNeasy-Kits (QIAGEN) and a sample without RNA, were used. First they were incubated for 5 minutes at 65°C and for 5 minutes at 41°C. Following this step, an enzyme mixture consisting of RNaseH, T7-polymerase and AMVV-RT was added and incubated for 90 minutes at 41°C. The amplified samples were subsequently placed on a non-denaturing gel and analyzed. For the 20 ng of total RNA isolated in the process described above, a specific transcript can be demonstrated (Figure 9).

Figure 9 shows an ethidium-bromide stained agarose gel of an electrophoretic separation of the NASBA-reactions.

Lanes 1 to 8: NASBA-Reactions:

Lanes 1 & 2: RNA from mouse liver purified from an aqueous solution with the 1.2 μm Hydrolon membrane;

Lane 3 & 4: RNA from HeLa-cells purified with the Sartolon membrane;

Lane 5 & 6: RNA from HeLa-cells purified with the 3 μm Hydrolon membrane;

Lane 7: RNA purified using the RNeasy-Mini-Kit;

Lane 8: Control without RNA.

Example 29: NASBA-reaction for amplification and detection of β-actin mRNA on hydrophobic membranes

According to the procedures of Example 1, plastic columns were assembled with commercially available membranes (Pall, Hydrolon with a pore size of 3 μm; Supor-450 PR with a pore size of 0.45 μm; Millipore, Fluoropore with a pore size of 3 μm).

For the isolation of RNA, different quantities of HeLa cells were used, the purification of total RNA was carried out as described in Example 3. The elution was performed by adding 20 μl NASBA-reaction buffer. The NASBA-reaction is subsequently performed on the membrane.

A NASBA-reaction is performed under standard conditions (Fahy, E. et al., 1991, *PCR Methods Amplic.*, 1:25–33). For amplification, β-actin specific primers were used.

The reaction device was first incubated for 5 minutes at 41°C in a water bath. Following this step, an enzyme mixture consisting of RNaseH, T7-Polymerase and AMVV-RT was added and incubated for 90 minutes at 41°C. The amplified samples subsequently were placed on a non-denaturing gel and analyzed. For the quantity of RNA used and isolated from 5×10^5 to 3×10^4 HeLa cells, a specific transcript can be observed for the total RNA isolated by the process described here.

Figure 10 shows an ethidium-bromide stained agarose gel of an electrophoretic separation of the NASBA-reactions.

Fig. 10A: Lanes 1 to 4: RNA from HeLa-cells purified with the 3 μm Hydrolon membrane:

Lane 1: 2.5 x 10^5 cells;

Lane 2: 1.25 x 10^5 cells;

Lane 3: 6 x 10^4 cells;

Lane 4: 3 x 10^4 cells.

Fig. 10B: Lanes 1 to 3: RNA purified from HeLa-cells:

Lane 1: RNA from 2.5 x 10^5 HeLa-cells purified with the 3 μm Hydrolon membrane;

Lane 2: RNA from 5 x 10^5 HeLa-cells purified with the Supor-450 PR membrane;

Lane 3: RNA from 5 x 10^5 HeLa-cells purified with the 3 μm Fluoropore membrane;

Example 30: Restriction of plasmid DNA with the *Ava* I enzyme on a hydrophobic membrane

According to the procedures of Example 1, plastic columns were assembled with hydrophobic membranes (e.g., Supor-200 PR from Pall).

100 μl of a plasmid-containing aqueous solution (pCMVβ by Clontech) were mixed with 350 μl of a lysis buffer containing guanidinium isothiocyanate (4 M GITC, 0.1 M $MgSO_4$, 25 mM sodium acetate, pH 4). Subsequently, 250 μl of isopropanol were added and mixed by pipetting. This mixture was then introduced to the column and passed through the membrane, washed and dried according to Example 4.

100 μl of a 1 x buffer for the restriction enzyme *Ava* I were placed on the membrane and either: (1) removed, transferred to a new reaction device and subsequently treated with the restriction enzyme (i.e., *Ava* I by Promega); or (2) a restriction enzyme (i.e., *Ava* I by Promega) was added directly to the eluate in the column.

The reaction mixtures were incubated for 1 hour at 37°C and subsequently placed on a non-denaturing gel and analyzed (see Figure 11).

Figure 11 shows an ethidium-bromide stained agarose gel of an electrophoretic separation of pCMVβ-plasmid after restriction with *Ava* I

Lane 1: uncut plasmid;

Lanes 2 & 3: elution with the reaction buffer for *Ava* I, restriction reaction in a separate device;

Lane 4 & 5: restriction with *Ava* I on the membrane.

Example 31: Pressure filtration for isopropanol precipitation of DNA

The isolation of plasmid DNA was performed according to standard protocols including the elution step via anion exchange chromatography. The DNA was eluted from the column in a high saline buffer.

Subsequently, 0.7 volume of isopropanol was added to this DNA solution, the sample was mixed and incubated for 1-5 minutes at room temperature. A 0.45 μm cellulose acetate filter with a 5 cm^2 surface in a filtration cartridge (standard installation for sterile filtration, e.g., Minisart by Sartorius) was used as a filtration installation. This filter was connected to a syringe from which the plunger has been removed first. The syringe was then filled with the DNA/isopropanol mixture and pressed through the filter with the syringe plunger. A high percentage of the DNA in this precipitate stays on the filter (i.e., cannot pass the pores).

The plunger was again removed from the syringe, was inserted again, and air was pressed through the filter. This step was repeated once or twice and serves to dry the membrane.

Subsequently, elution was performed with a corresponding volume of a low saline buffer, whereby the buffer fills the syringe and was pressed through the filter with the plunger. To increase the yield, this first eluate was again put into the syringe and pressed through the filter with the plunger. In this test configuration, the yields obtained typically range from 80 to 90% (see Example 34).

Example 32: Vacuum filtration for the isopropanol precipitation of DNA

As with pressure filtration, first plasmid DNA was isolated and mixed with 0.7 volume isopropanol. An apparatus designed for vacuum filtration was used as a filtration installation, in which a 0.45 μm cellulose acetate filter with a surface of 5 cm^2 was placed. 0.45 μm cellulose nitrate filters or several layers of 0.65 μm cellulose acetate or cellulose nitrate filters may be used. The isopropanol-DNA mixture was incubated for 1-5 minutes and placed on the filter assembly. By creating a vacuum, the solution was suctioned through the filter. The DNA-precipitates on the filter were mixed with a corresponding volume of 70% ethanol and washed by creating a vacuum. The elution of the DNA from the filter takes place by adding a low salt buffer, a short incubation and renewed creation of a vacuum. The yield can either be obtained by

repeated elution from the filter with a second volume of low saline buffer or by elution with the eluate from the first elution step. Here also, typical yields range from 80% - 90% of the DNA.

Example 33

The method used is the vacuum filtration method described in Example 32. The filter device used is the vacuum filter apparatus, Sartorius 16315. pCMVβ was used as the plasmid DNA, which was isolated from DH5α cells.

Procedure: In each test, 15 ml of QF-buffer (high saline buffer) are mixed with 500 µg of plasmid. 10.5 ml of isopropanol are added and this is mixed again. Then the mixture is left to incubate for 5 minutes. The plasmid DNA thus precipitated is deposited on the membrane in the filter assembly. Next a vacuum is created and the filtration takes place. The membranes are washed with 5 ml of 70% ethanol (by creating another vacuum), then 1 ml TE-buffer is pipetted onto the membranes, left to incubate for 5 minutes, and the DNA is eluted by creating a vacuum. Subsequently a post-elution is performed with 1 ml TE-buffer. Total DNA amounts are measured in the flow-through, in the washing stage and in the combined eluate (OD260). The following results were obtained:

Membrane	Test Number	Flow-through	Washing Stage	Eluate	Flow Speed
PVDF 0.2 µm	1	0 µg DNA	0 µg DNA	131 µg DNA	Very slow
Cellulose Nitrate 0.65 µm	2	0 µg DNA	0 µg DNA	418 µg DNA	Fast
Cellulose Acetate 0.65 µm	3	0 µg DNA	0 µg DNA	469 µg DNA	Fast

Calculated on the basis of 500 µg of DNA starting quantity, the following yields are obtained with this method:

PVDF 0.2 µm	26%
Cellulose Acetate 0.65 µm	94%
Cellulose Nitrate 0.65 µm	84%

Example 34

The pressure filtration method indicated in Example 31 was used. The filter assembly used was a commercially available 0.45 μm cellulose acetate filter (Minisart, Sartorius). pCMV β is used as plasmid DNA, which was isolated from DH5α cells.

Procedure: For each test, 15 ml of QF-buffer (high salt buffer) are added to and mixed with 100, 200, 300, etc., up to 900 μg of plasmid. 10.5 ml isopropanol are added and again mixed. Subsequently, there is a 5-minute incubation period. The plasmid DNA thus precipitated is transferred to a syringe, to which the filter had been previously fitted. Pressure filtration takes place with the aid of the syringe. The filter is then washed with 2 ml of 70% ethanol and, as described, dried twice. The elution is performed with 2 ml of TE-buffer. A second elution is performed with the eluate. The total amount ofDNA is measured in the combined eluate (OD260).

Following the above procedure, the following results were obtained:

DNA-quantities used	DNA-quantities eluted	% Yield
100 μg	100 μg	100%
200 μg	176 μg	88%
300 μg	257 μg	86%
400 μg	361 μg	90%
500 μg	466 μg	93%
600 μg	579 μg	97%
700 μg	671 μg	96%
800 μg	705 μg	88%
900 μg	866 μg	96%

Example 35

The vacuum filtration method indicated in Example 32 was used. The filter assembly used was a commercially obtained 0.45 μm cellulose acetate filter (Minisart, Sartorius), that had been attached to a filtration chamber (QIAvac). As buffer reservoir, a syringe was attached to the other end of the filter. pCMVβ was used as plasmid DNA, which was isolated from DH5α cells.

Procedure: 15 ml of QF-buffer (high saline buffer) are added to and mixed with 500 µg of plasmid. 10.5 ml isopropanol are added and again mixed. Subsequently, there is a 5-minute incubation period. The plasmid DNA thus precipitated is then transferred to the filter assembly syringe. Now a vacuum is created and filtration takes place. The filter is not washed with 70% ethanol. Rather, elution with 2 ml of EB buffer (QIAGEN) follows immediately. Post-elution is performed with the eluate. The total DNA quantity in the combined eluate is measured (OD260). The following result was obtained:

Test Number	Eluted DNA	% Yield
1	434 µg	87%
2	437 µg	87%

Although a number of embodiments have been described above, it will be understood by those skilled in the art that modifications and variations of the described devices and methods may be made without departing from concept of the invention as defined in the appended claims. The articles and other publications cited herein are incorporated by reference.